Universidade de Aveiro Secção Autónoma das Ciências da Saúde
2012

Maria João Martinho de Freitas

Identificação do interactoma da TCTEX1D4 no testículo humano.

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Professora Doutora Margarida Sâncio da Cruz Fardilha, Professora Auxiliar Convidada da Secção Autónoma das Ciências da Saúde da Universidade de Aveiro

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palavras-chave

TCTEX1D4, PPP1, interactoma, espermatozoide, testículo humano, dois híbrido levedura.

resumo

T-complex testis expressed protein 1 domain containing 4 (TCTEX1D4) é uma cadeia leve de dineina identificada como sendo uma proteina que interage, no testículo humano, com a fosfoproteina fosfatase 1. As funções específicas da TCTEX1D4 ainda são desconhecidas e identificar as suas proteinas interactoras pode ilucidar sobre as funções desta. Foi aplicado o método de dois híbrido de levedura com o intuito de identificar o interactoma da TCTEX1D4. Foram obtidos 494 clones positivos, dos quais 86 foram identificados correspondendo a 44 diferentes proteinas. Uma análise *in silico* das características funcionas de todas as proteinas identificadas revelou que as proteinas que interagem com a TCTEX1D4 apresentam funções tão diversas como ligação a iões, ligação ao DNA e actividade peptídica. Também foram obtidas os padrões de expressão em diversos tecidos da base de dados UniGene. Duas proteinas que interagem com TCTEX1D4 são específicas de testículo enquanto 5 são enriquecidas nestes tecido. A rede de interação da TCTEX1D4 foi construída no Cytoscape e combinando os padrões de expressão foi possível identificar possíveis complexos proteicos da TCTEX1D4 específicos ou enriquecidos no testículo. Os complexos TCTEX1D4/TCTEX1D2 e TCTEX1D4/CRISP2 foram caracterizados mais profundamente, revelando que a TCTEX1D4 pode estar envolvida na reação acrossómica, mobilidade do espermatozoide e na interação célula-célula. Em conclusão as funções da TCTEXD4 ainda são desconhecidas mas a identificação e caracterização do seu interactoma ajuda a revelar as suas possiveis funções.

keywords

TCTEX1D4, PPP1, interactome, spermatozoa, testis, network, yeast two hybrid.

abstract

T-complex testis expressed protein 1 domain containing 4 (TCTEX1D4) is a dynein light chain that was identified as a phosphoprotein phosphatase 1 interacting partner in human testis. The specific functions of TCTEX1D4 in testis are still unknown and identification of TCTEX1D4 interacting proteins can ilucidate possible functions of this protein. A yeast two hybrid approach was undertook to identify the TCTEX1D4 interactome. We obtained 494 positive clones from which 86 clones were identified corresponding to 44 different proteins. An *in silico* analyzis was performed for all proteins identified. *In silico* functional characterization of TCTEX1D4 interactome reveald its diverse in cellular functions ranging from proteins with ion binding function to DNA binding and peptidase activity. Also a tissue expression distribution was obtained from UniGene database and 2 specififc testis and 5 testis enriched TCTEX1D4 interacting proteins were identified. A TCTEX1D4 network was constructed n Cytoscape and combining tissues expression profiles we were able to identified possble TCTEX1D4 protein complexes specific or enriched in testis. Two TCTEX1D4 protein complexes TCTEX1D4/TCTEX1D2 and TCTEX1D4/CRISP2 were further studied revealing that TCTEX1D4 may be involved in acrosome reaction, sperm motility and cell-cell interaction. In conclusion TCTEX1D4 is still known but identification and characterization of its interactome can help unveil its putative functions.

Index

Abreviations 3

I. Introduction 5

I.1. Overview on the Male Reproductive System 7

I.2. Testis: structure and function 7

I.3. Spermatozoa: structure and function 8

I.4. Spermatogenesis and spermatozoa maturation 8

I.5. Reversible phosphorylation: Protein Phosphatases 10

I.6. PPP1 and phosphatase interacting proteins 11

I.7. TCTEX1D4: A novel PPP1 interacting protein in testis and sperm 13

I.8. Yeast Two Hybrid System: Unveiling protein-protein interactions 20

II. Aims 23

III. Methods and Materials 27

III.1. Library construction and selection of the positive clones 29

III.2.Plasmid isolation from yeast 29

III.3. Bacterial transformation 30

III.4. Plasmid analysis 30

III.5. Sequence analysis – Bioinformatics 33

IV. Results and Discussion 35

IV.1. Identification of the Yeast Two Hybrid positive clones 37

IV.2. Analysis of the positive clones 39

IV.3. TCTEX1D4 interacting proteins tissue distribution 47

IV.4. Functional characterization of the human testis TCTEX1D4 Interactome 53

IV.5. TCTEX1D4 Network 55

IV.6. TCTEX1D4/TCTEX1D2 complex 59

IV.7. TCTEX1D4/CRISP2 complex 62

V. Conclusion 69

VI. References 73

VII. Appendix 82

VII.1. Culture Media and Solutions.....83
VII.2. Yeast Strains and Bacteria.....86
VII.3. Plasmids 87
VII.4. Primer 89

Abreviations

aa	Amino acid(s)
AD	Activation Domain
Ade	Adenosine
Amp	Ampicillin
BD	Binding domain
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary deoxynucleic acid
CDS	Coding sequence
Chr	Chromosome
CRISP2	Cystein-rich secretory protein 2
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
DLC	Dynein light chain
DNA	Deoxynucleic acid
dNTP	Deoxynucleotide triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
EDTA	Ethylenediaminotetraacetic acid
GAL4	Gal4 transcription factor
GAL4-AD	GAL4-Activation domain
GAL4-BD	GAL4-Binding domain
IFT	Intraflagellar transport
LB medium	Luria-Bertani Medium (Miller)
LB	Loading Buffer
Nt	Nucleotide
OD	Optical density
ORF	Open Reading frame
PCR	Polimerase chain reaction
PPP1	Phospho Protein Phosphatase 1
PPP1c	Phospho Protein Phosphatase 1 catalytic subunit
QDO	Quadruple dropout
RNA	Ribonucleic acid

RT	Room Temperature
SD	Supplement dropout medium
SDS	Sodium dodecyl sulfate
TCTEX1D2	T-complex testis expressed protein 1 containing domain 2
TCTEX1D4	T-complex testis expressed protein 1 containing domain 4
TDO	Triple dropout
UAS	Upstream activating sequence
UV	Ultraviolet
YTH	Yeast two Hybrid

I. INTRODUCTION

I.1. Overview on the Male Reproductive System

The male reproductive system consists of two testes, a system of genital ducts, the accessory glands and the penis. It has the purpose of producing spermatozoa (spermatogenesis) and male sexual hormones. When fully functional, the male reproductive system is capable of delivering spermatozoon into the female reproductive tract and fertilizing the oocyte (Vander *et al.*, 2001).

I.2. Testis: structure and function

Testis is the male gonad in mammals. It has two roles: produce spermatozoa and produce hormones. The production of both products occurs in two discrete compartments. Spermatozoa develop within the seminiferous tubules in close association with Sertoli cells while hormones, mainly testosterone, is synthesized between the tubules in the Leydig cells (Johnson, 2007). In figure 1 is a schematic representation of the testis.

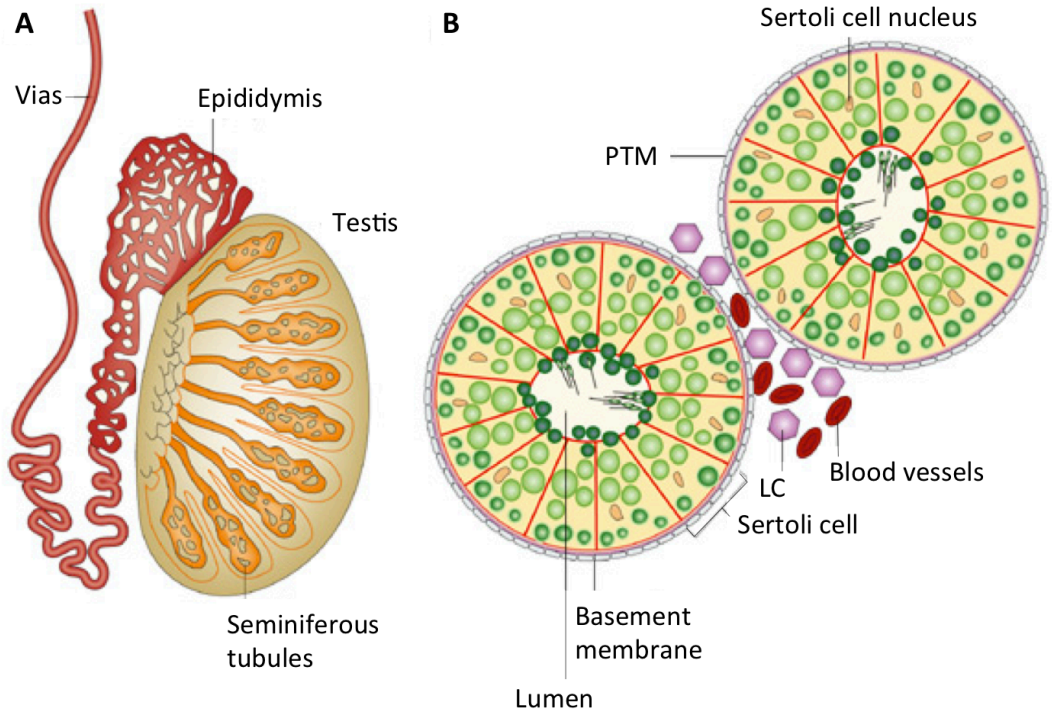


Figure 1. Testis structure. **A.** Crossection of testis showing seminiferous tubules, the epididymis and the vas deferents. **B.** Crossection of a testicular tubule, showing germ cells in different stages of maturation embedded in Sertoli cells. In the interstitium are de Leydig cells (adapted from Cooke *et al.*, 2002)

I.3. Spermatozoa: structure and function

The male germ cell, the spermatozoon, is a highly specialized cell, which has the solo purpose of crossing the female reproductive tract to fertilize the oocyte. The spermatozoon is composed of a head and tail. Each component has special characteristics in order to perform specific functions (Sutovsky *et al.*, 2006). The head is formed by the acrosome vesicle, nucleus, cytoskeletal structures and cytoplasm (Reid *et al.*, 2011). It carries the genetic information and is responsible for the acrosome reaction. The tail can be further divided in: midpiece, principal and end pieces (Sutovsky *et al.*, 2006). The midpiece houses the mitochondria, which are responsible for the energy production. The principal and end pieces are responsible for the movement and progression of spermatozoa (Reid *et al.*, 2011). Spermatozoa components are represented in figure 2.

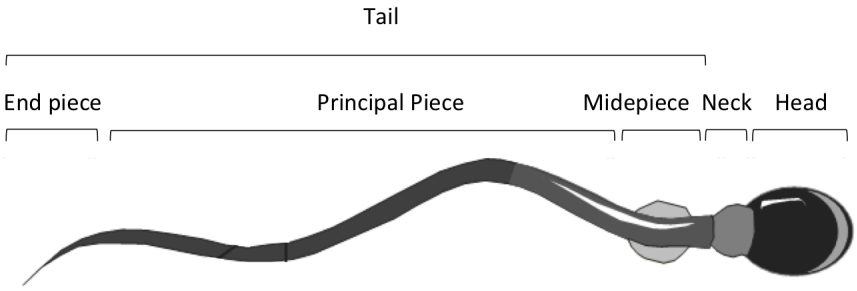


Figure 2. **Spermatozoa components** (adapted from Fardilha *et al.*, 2011a).

I.4. Spermatogenesis and spermatozoa maturation

Spermatozoa are produced through a process called spermatogenesis, by meiosis, in the male gonads (testes). Spermatogonia are undifferentiated cells, which suffer several cell divisions and some differentiation processes, resulting in primary spermatocytes. Then, each spermatocyte formed increases markedly in size and undergoes the first meiotic division (secondary spermatocyte) followed by a second meiotic division (spermatid). The final phase of spermatogenesis is the differentiation of spermatids into spermatozoa. This involves extensive cell remodeling (Vander *et al.*, 2001). By the end of spermatogenesis, spermatozoa are morphologically mature but functionally incomplete and are incapable to perform progressive motility and interaction with the *zona pellucida* (ZP) of the oocyte (Reid *et al.*, 2011).

Spermatozoa epididymal maturation is a progressive process in which functional attributes are acquired while spermatozoa crosses the epididymis from *caput* to *cauda*. Interestingly, changes in gene expression are not responsible for functional modifications since transcription in spermatozoa is impaired, mainly because of DNA packing by protamines, which replace the histones in the late stages of spermatogenesis (Reid et al., 2011). Therefore, there are intracellular control mechanisms responsible for functional modifications, such as reversible phosphorylation of structural and regulatory proteins. The epididymal luminal milieu, in which the spermatozoa are bathed, contribute to the functional acquisition, for example, by bulk transfer of proteins from the epididymal lumen to the sperm cell, although the exact mechanisms are still unknown (Reid *et al.*, 2011; Vieira *et al.*, 2011). It has been proposed that primary regulation of flagellar beating that happens in the epididymis occurs through reversible phosphorylation of axonemal proteins (Fardilha *et al.*, 2011a; Publicover *et al.*, 2011).

The final stage of sperm maturation is the capacitation. It takes place in the female reproductive tract, after ejaculation. Biochemical and biophysical changes stimulate a panoply of signal transduction pathways, that ultimately have the purpose of hyperactivating motility, enabling the recognition of ZP by the spermatozoa and acrosomal exocytosis (Reid *et al.*, 2011). In Figure 3 are represented the changes in sperm morphology and motility throughout spermatogenesis, epididymal maturation and capacitation.

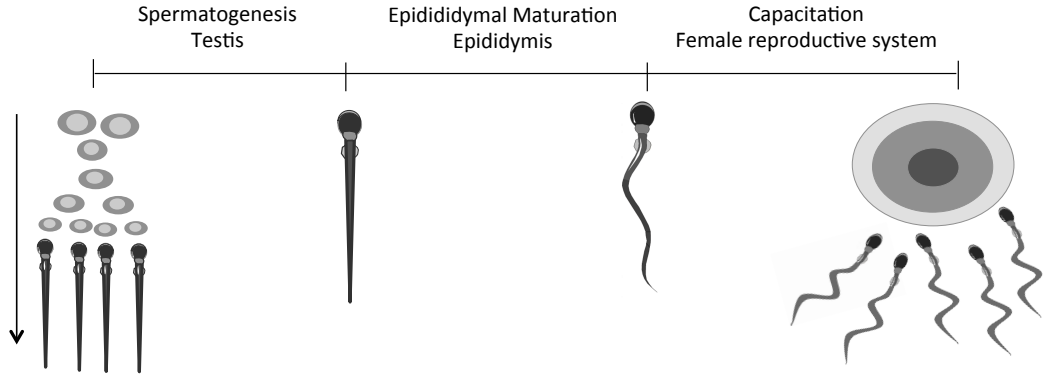


Figure 3. Scheme representing the distinct changes in sperm morphology and motility throughout spermatogenesis, epididymal maturation and capacitation. Spermatogenesis, which occurs in testis, comprises a sequence of cell divisions parallel to drastic morphological alterations. Following spermatogenesis, spermatozoa undergo epididymal maturation. By the end of this stage the spermatozoa are able to move forward. In the final stage of maturation, capacitation, complex signaling pathways are activated that result in hyperactivated motility and ability to bind the ZP (adapted from Reid *et al.*, 2011)

I.5. Reversible phosphorylation: Protein Phosphatases

Reversible phosphorylation of structural and regulatory proteins is an essential intracellular control mechanism in eukaryotes, being involved in several functions such as, signal transduction, cell division and memory. It involves both protein kinases (that add a phosphate group) and protein phosphatases (that remove a phosphate group). The number of human diseases that arise by impaired control of protein phosphorylation demonstrates the importance of such metabolic control mechanism. Cancer, diabetes and neurodegenerative conditions are a few examples. (Fardilha *et al.*, 2010).

Protein phosphatases are divided in 2 main groups (table 1): protein tyrosine phosphatases (PTPs) and serine/threonine specific protein phosphatase (STPPs). PTPs dephosphorylate phospho-tyrosine residues and dual specificity phosphatases, a family of PTPs, catalyze the dephosphorylation of both phospho-tyrosine and phospho-serine/threonine residues. There are 3 STPPs gene families. metal–dependent protein phosphatase (PPM) that needs Mg^{2+} or Mn^{2+}; the aspartate-based phosphatases, which are represented by the FCP/SCP family and have the terminal domain of RNA polymerase II as a substrate and the Phosphoprotein Phosphatase (PPP) family. The last one is composed by PPP1, PPP2/4/6, PPP3 and PPP5-7 subfamilies that share high homology in the catalytic domain but the C and N terminal are distinct (Cohen, 1997; Fardilha *et al.*, 2011b).

Table 1. Protein Phosphatase families. (adapted from Cohen, 1997; Fardilha *et al.*, 2010; Fardilha *et al.*, 2011a; Fardilha *et al.*, 2011b; Korrodi-Gregório, 2012).

GROUP	FAMILY	SUBFAMILY/ MAJOR MEMBERS
Protein tyrosine phosphatase (PTP)	Classical PTP	Non-transmembranar PTP
		Receptor-type PTP
	Dual specificity PTP	MAP kinase phosphatase 1
Serine/threonine specific protein phosphatase (STPP)	Metal – dependent protein phosphatase (PPM)	Pyruvate dehydrogenase PPP2C
	Transcription factor IIF associating C-terminal domain phosphatases/ small CTD phosphatase (FCPs/SCP)	FCP1 and SCPs
	Phosphoprotein phosphatase (PPPs)	PPP1
		PPP2/4/6
		PPP3

GROUP	FAMILY	SUBFAMILY/ MAJOR MEMBERS
		PPP5/7

PPP1 is the main protein phosphatase and is expressed in all eukaryotic cells. It exists as a complex heterodimer consisting of a catalytic and regulatory subunit. In mammals, PPP1 catalytic subunit (PPP1C) is encoded by three different genes: PPP1CA, PPP1CB and PPP1CC. PPP1CC by alternative splicing can form PPP1CC1 and PPP1CC2, which differ exclusively at the C-terminus. Each isoform has a unique tissue distribution and cellular localization. Tissue expression of PPP1 subfamily is shown in table 2.

Table 2.Tissue expression of PPP1 subfamily (adapted from Cohen, 2002; Fardilha *et al.*, 2011a; Korrodi-Gregório, 2012).

PROTEIN PHOSPHATASE	TISSUES
PPP1CA	Ubiquitous, but abundant in brain
PPP1CB	Ubiquitous, but abundant in liver and kidney
PPP1CC1	Ubiquitous, but abundant in brain, small intestine and lung
PPP1CC2	Testis-enriched and sperm-specific. Low abundance in other tissues.

Interestingly, protein phosphatases have fewer encoding genes (2-5 times less), when comparing with protein kinases. The diversity of catalytic and regulatory subunits may explain the imbalance between protein kinases and phosphatases. However, it has became apparent that the versatility of PPP1 is achieved by the interaction with a panoply of proteins, named phosphatase interacting proteins, or PIPs (Fardilha *et al.*, 2011a).

I.6. PPP1 and phosphatase interacting proteins

More than 200 PIPs have already been identified but many more remain unknown (Fardilha *et al.*, 2011a). Almost all PIPs have the consensus PPP1 binding motif, the RVxF that binds to the PPP1 catalytic subunit (Hendrickx *et al.*, 2009; Korrodi-Gregório, 2012). Crystallographic studies showed that RVxF binds as a β strand to a hydrophobic groove of PPP1 located distantly from the catalytic site (Bollen *et al.*, 2010). Binding of the docking motif of PIPs does not change the PPP1 catalytic subunit conformation and localization. However, it is essential because it promotes the occupation of other lower affinity binding sites, present in the PIP, leading to changes in activity and substrate specificity of PPP1 (Bollen, 2001; Hendrickx *et al.*, 2009; Bollen *et al.*, 2010).

Each PIP or set of PIPs is fundamental for PPP1 function, being responsible for determining its function and specific cellular function. It has been already proved the PPP1 involvement in a variety of processes, such as cytoskeleton functions (Kao *et al.*, 2007), spermatozoa motility (Fardilha *et al.*, 2011a), meiosis and apoptosis (Cohen, 2002). Clearly, the diversity of PPP1 function is achieved by the capacity to form distinct complexes with different PIPs and therefore characterizing novel roles of PPP1 will only be possible with the discovery of novel PIPs (Fardilha *et al.*, 2011a; Korrodi-Gregório, 2012).

I.6.1. PPP1CC2 complexes in spermatozoa motility

PPP1CC2 is an isoform of PPP1, that is testis-enriched and sperm-specific (Kitagawa *et al.*, 1990; Vijayaraghavan *et al.*, 1996; Fardilha *et al.*, 2011a). In testis, PPP1CC2 appears to be involved in sperm differentiation and morphogenesis, since a *Ppp1cc* gene null male mice is infertile due to impaired spermatogenesis (Varmuza *et al.*, 1999; Chakrabarti *et al.*, 2007). In spermatozoa, PPP1CC2 is present in the flagellum and head, suggesting a role in spermatozoa motility and acrosomal reaction (Huang *et al.*, 2002; Chakrabarti *et al.*, 2007). Moreover, PPP1CC2 activity is higher in immotile spermatozoa, compared to motile (Smith *et al.*, 1996; Vijayaraghavan *et al.*, 1996).

The new accepted model proposes that a signaling transduction mechanism relying on PPP1CC2 and its PIPs could control the acquisition of spermatozoa motility along the epididymis. The model states that when PPP1CC2 is activated it dephosphorylates several spermatozoa proteins and promotes immotility in the caput of epididymis. When PPP1CC2 is inhibited, several spermatozoa proteins are phosphorylated and the spermatozoon is capable of acquire motility. Several complexes of PPP1CC2 and its PIPs are suggested to modulate PPP1CC2 activity, such as PPP1CC2/PPP1R2/GSK3, PPP1CC2/PPP1R2P3/GSK3 PPP1CC2/PPPR11, PPP1CC2/PPP1R7 and PPP1CC2/AKAP/PKA (Fardilha *et al.*, 2011a). Figure 4 illustrates the effects of PPP1CC2 activity in sperm motility.

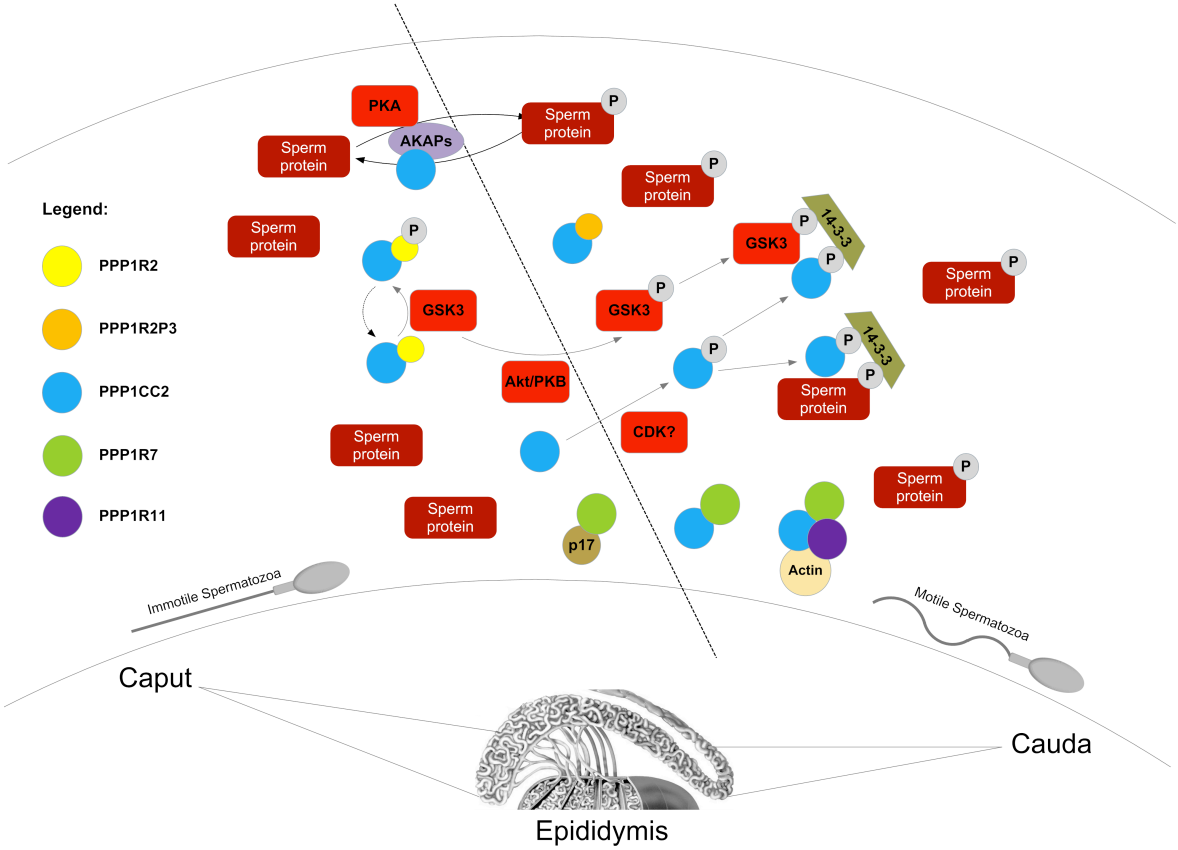


Figure 4. Illustrative mechanism of sperm motility acquisition based on PPP1CC2 regulation. PPP1CC2 is inhibited by PPP1R2 that is phosphorylated by GSK3 releasing active PPP1CC2 in the caput epididymis. In the cauda of epididymis, PPP1CC2 is bound to PPP1R2P3 that cannot be phosphorylated by GSK3. The protein 14-3-3 binds PPP1CC2 and GSK-3 and also other sperm phosphoproteins in the caudal epididymis. Inhibition of PPP1CC2 or activation of PKA through AKAP anchoring will induce sperm motility via phosphorylation of various sperm proteins. The signals underlying these events are still unknown. PPP1CC2 may also be inhibited by phosphorylation in a C-terminal threonine residue by a CDK that still needs to be identified. PPP1R7 binding to PPP1CC2 inhibits its activity in the cauda of epididymis. In the caput epididymis, PPP1R7 is unable to inhibit PPP1CC2 since it is bound to the protein p17. Also, a multimeric complex has been identified composed by PPP1CC2, PPP1R7, actin and PPP1R11, where PPP1CC2 is inactive. (Fardilha *et al.*, 2011a)

I.7. TCTEX1D4: A novel PPP1 interacting protein in testis and sperm

Yeast two-hybrid screen from a humam testis cDNA library, identified the t-complex testis expressed protein 1 domain containing 4 (TCTEX1D4) as a novel PPP1CC2 interacting protein (Korrodi-Gregório, 2012). This protein has been recently described as a novel DYNLT1/TCTEX1 dynein light chain family member (Meng *et al.*, 2006). For a better understanding of TCTEX1D4 cellular functions, the next sections will provide a brief summary of dynein structure and function.

I.7.1. Dyneins: structure and function

Eukaryotic cells need to organize their cytoplasm by moving cargo, normally organelles and macromolecular complexes, along the microtubules. These movements are essential for several processes, such as mitosis, signal transduction and maintenance of cellular environment. This movement can be anterograde, when performed by the action of protein known as kinesins, moving the cargo towards the cell periphery or retrograde, performed by proteins known as dyneins and in the opposite direction. Microtubules are also found in cellular specialized components, cilia and flagella, in a common structure denominated axoneme (Alberts *et al.*, 2002). In spermatozoa, microtubules are the main component of axonemal flagella and therefore responsible for the tail beating necessary for motility (Inaba, 2003).

Dyneins are molecular motors that use ATP hydrolysis to power up a variety of cellular functions. They are multimeric complexes composed of 4 subunits: dynein heavy chains (DHCs), intermediate chains (DICs), light intermediate chains (DLICs) and light chains (DLCs). In order to achieve useful and diverse work, dynein subunits must perform 3 tasks: motion, regulation of motion and cargo-binding (King, 2000). The most intriguing aspect of dynein is the ability of connecting to a multitude of cargos with high specificity within a single cell (King, 2000). Dynein subunits and their functions are summarized in table 3.

Table 3. Dynein subunits and their functions.

SUBUNIT	FUNCTIONS	REFERENCE SUPORTING THE FUNCTION
Heavy Chains	Responsible for cargo movement from the cell periphery toward the nucleus. It binds reversibly to microtubule and is capable of converting chemical energy (ATP) into movement. Is an ATPase Associated with diverse cellular Activities (AAA) protein.	Karcher *et al.*, 2002; Sakakibara *et al.*, 2011
Intermediate Chains	Mediates cargo binding to dynein and regulates the motion by altering motor activity.	King, 2000; Karcher *et al.*, 2002; Hook *et al.*, 2006
Light intermediate Chains	Mediates cargo binding to dynein.	Karcher *et al.*, 2002
Light Chains	It is the cargo binding subunit. It can do it directly or through	Karcher *et al.*, 2002

SUBUNIT	FUNCTIONS	REFERENCE SUPORTING THE FUNCTION
	binding partners (for example dynactin). Due to the fact that members of this group are quite divergent in the tail domain, they bind to a wide a variety of cargo.	

According to cell localization, functional and structural characteristics, dyneins can be classified as axonemal or cytoplasmic.

Cytoplasmic dyneins are abundant and associated with the transport of a variety of cellular components, such as, elements of the Golgi apparatus and lysosomes. Due to the diversity of possible cargos, dyneins are involved in different processes such as mitosis and embryonic development and have an important role in axonal transport and in intraflagellar transport (transport of cargo along the axoneme) (Hook *et al.*, 2006; Meng *et al.*, 2006; Korrodi-Gregório, 2012).

Axonemal dyneins are responsible for ciliar and flagellar whipping. The basic structure of axonemes consists of 20 microtubules: two centrally located and nine arranged in pairs forming a surrounding cylinder. Axonemes bend when dyneins on one side are active while those on the other side are inactive. Dyneins on the active side 'walk' towards minus ends, powering active sliding between doublets. On the inactive side, dyneins move passively in the opposite direction. To propagate a beat down of the axoneme, the dyneins activity must be spatially and temporally tightly controlled (Hook *et al.*, 2006; Mitchison *et al.*, 2010). Axonemal dyneins, regarding their spatial orientation, can be divided in inner dynein arm and outer dynein arm. While inner arm and cytoplasmic dynein only have two motor heads, the outer arm dynein has three motor heads (Hook *et al.*, 2006). Also outer dynein arms determine the maximal velocity of outer doublet microtubule sliding while inner dynein arms affect flagellar wave form, regulate beating symmetry and/or provide additional force need to maintain a high velocity (Gagnon *et al.*, 2006). Figure 5 illustrates cytoplasmic dynein and an axoneme crossection.

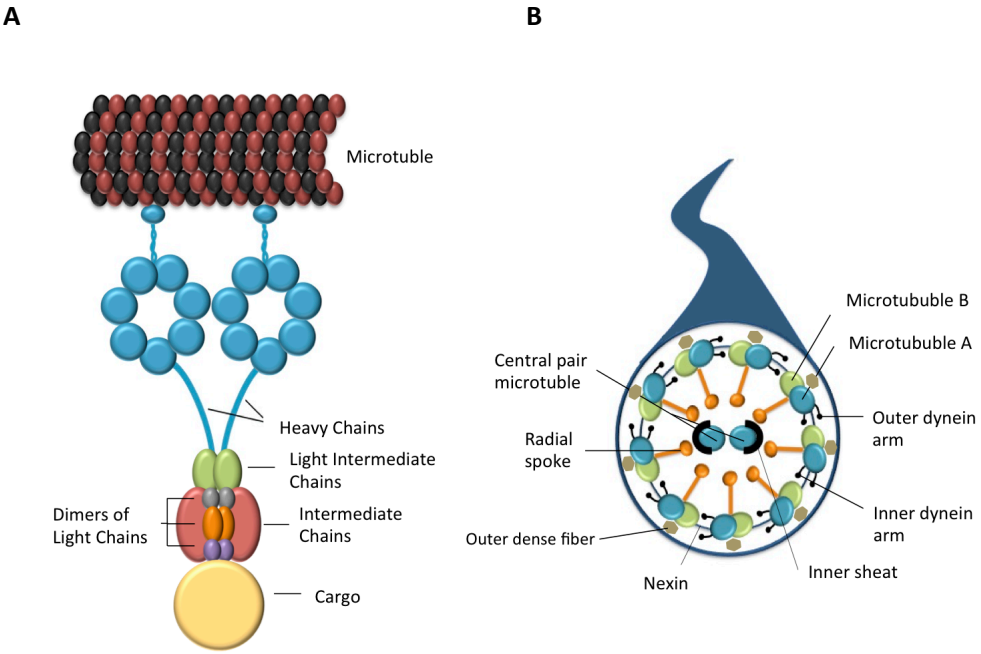


Figure 5. **Illustrative structure of cytoplasmic dynein and axoneme crossection from spermatozoa**. **A.** Heavy chains (in blue) homodimerize in the N terminal region and in the C terminal form the motor domain (7 large globular heads). The microtubule-binding domain is projected from the heavy chain. Two intermediate chains and two light intermediate chains bind at overlapping regions of the N-terminus of the heavy chain. Dimers of the three light chain families bind to the intermediate chain dimers. **B.** Microtubule doublets are shown as overlapping circles (blue and green), the central pair of microtubule is in blue and dynein arms are in black. Dynein connects the outer pairs and forces them to slide against each other.

I.7.2. TCTEX1D4, DYNT1/TCTEX1 family member

Dynein light chains are a superfamily of proteins that are responsible for cargo binding specificity. There are 3 families of DLC proteins: LC8, Roadblock/LC7 and DYNT1/TCTEX1 (King, 2000; Meng *et al.*, 2006).

TCTEX1D4 is a novel dynein light chain protein of the DYNT1/TCTEX1 family. It is localized to chromosome 1p34.1, has 2 exons and 221 amino acids. Structurally, TCTEX1D4 has 2 domains: the disordered (residues 1-120) and the globular (residues 121-221). The globular domain is common to all family members (Meng *et al.*, 2006). Also, TCTEX1D4 gene belongs to t- complex. In mouse, the t-complex is a naturally occurring variant part of the chromosome 17 and is characterized by several gene inversions. Different variants of the t-complex exist, the t-haplotypes, and differ among them by extend of the inversions and the consequent embryonic gene lethality. It seems that the inversions have been maintained in t-haplotypes because they lock together a set o genes essential for male fertility. The large DNA inversions present in the t- complex dramatically reduce the rate of crossing over between wild-type and t- complex chromosome (Fraser *et al.*,

1999).

By bioinformatic approaches, Korrodi-Gregório *et al* (2012) revealed that TCTEX1D4 contains many putative serine phosphorylation sites, mainly in the disordered domain, for several kinases, such as protein kinase A, B and C, cyclin dependent kinase 1 and glycogen synthase kinase 3. These putative phosphorylation sites support the involvement of TCTEX1D4 in cell cycle, proliferation, differentiation and metabolism. Curiously, potential threonine and tyrosine phosphorylation sites were not found. As expected, the consensus PPP1 binding motif, RVxF, was found in residues 90-93, reinforcing the idea that TCTEX1D4 is a PIP. The fact that serine phosphorylation appears to be the frequent post-translational modification supports the role of PPP1 as a control mechanism for TCTEX1D4 function (Korrodi-Gregório, 2012). The schematic representation of human TCTEX1D4 protein is illustrated in figure 6.

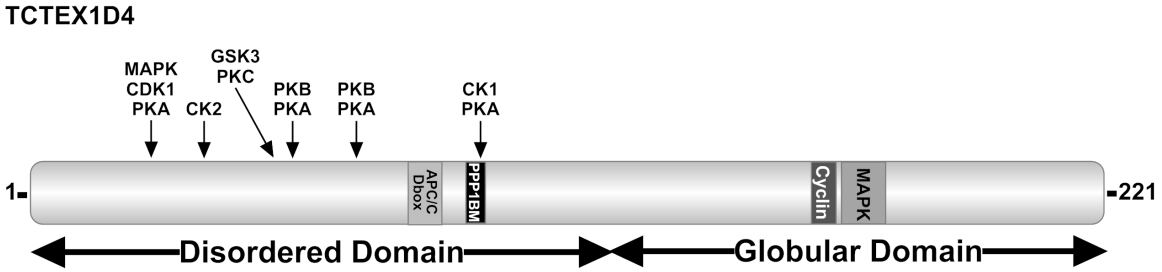


Figure 6. Schematic representation of human TCTEX1D4 protein. Disordered and globular domains are presented. Putative serine phosphorylation sites for protein kinase A (PKA, Ser24/53/66/92), B (PKB, Ser53/66) and C (PKC, Ser49), cyclin dependent kinase 1 (CDK1, Ser24), casein kinase 1 (CK1, Ser92) and 2 (CK2, Ser34), mitogen-activated protein kinase (MAPK, Ser24), and glycogen synthase kinase 3 (GSK3, Ser49) are showed. The PPP1 binding motif (PPP1BM) is also showed. APC/C Dbox: anaphase promoting complex destruction box (Korrodi-Gregório, 2012).

Being a DLC, TCTEX1D4 confers specificity to cargo binding and along with DIC forms the cargo complex. Both DIC and TCTEX1D4 may be dephosphorylated by PPP1 and therefore regulate their function by reversible phosphorylation (Korrodi-Gregório, 2012).

It appears that TCTEX1D4 is involved in the TGFβ (transforming growth factor beta) signaling by interacting with endoglin, a TGFβ type III receptor. It increases the retention time of endoglin and TGFβ type III receptor in de cell membrane preventing ligand induced signaling. (Meng *et al.*, 2006).

I.7.3. TCTEX1D4: tissue distribution and subcellular localization

TCTEX1D4 was found to be present in a variety of tissues. In rat, the tissues with the highest level of TCTEX1D4 expression are the ovary, spleen, lung, placenta and kidney. In human testis,

TCTEX1D4 is expressed at low levels. In mouse testis, TCTEX1D4 is expressed in all germ cell stages and in Sertoli (spermatozoa supporting cells) and Leydig (androgen producing cells) cells. Particularly, in late germ cells, TCTEX1D4 is enriched in microtubules and MTOC (microtubule organizing center). In Sertoli and Leydig cells TCTEX1D4 is enriched in the blood testis barrier (BTB). This pattern of expression suggests that TCTEX1D4 is involved in the regulation of BTB selectivity, mainly through the TGFβ pathway, and in intracellular trafficking (Meng *et al.*, 2006; Korrodi-Gregório, 2012).

Regarding spermatozoa, TCTEX1D4 is present in all regions, but is more prominent in the tail and in the midpiece region. However in the tail is uncertain whether it has a role in the axonemal dynein promoting motility or/and as a cargo binding protein in intraflagellar transport. This type of subcellular localization confirms that TCTEX1D4 is a DLC with both cytoplasmic and axonemal functions (Korrodi-Gregório, 2012).

I.7.4. TCTEX1D4 as testis and spermatozoa PPP1 interacting protein.

In mammalian cells, PPP1 and TCTEX1D4 co-localize in microtubules and MTOC. It seems that TCTEX1D4 may regulate PPP1 function, being at least partially responsible for it´s retrograde transport to specific subcellular regions (Korrodi-Gregório, 2012). Fine-tuning of PPP1 microtubule localization is important to regulate processes, which depend on microtubule dynamics, such as mitosis (Tournebize *et al.*, 1997). The presence of TCTEX1D4 in microtubules and MTOC agrees with its function as a DLC and implies an essential role in microtubule organization, dynamics and MTOC cellular positioning (Gonczy *et al.*, 1999).

In human spermatozoa, localization pattern of TCTEX1D4 and PPP1CC2 suggests a co-localization of these two proteins, being the pattern stronger in flagellum and weaker in the head (Korrodi-Gregório, 2012). Such distribution hints a role for TCTEX1D4-PPP1CC2 holoenzyme in sperm motility. PPP1CC2 can alter the dynein function in axoneme by dephosphorylating TCTEX1D4 or other component of the dynein arm and/or is a TCTEX1D4 cargo being transported in a retrograde manner to specific locations within flagellum in order to perform its role, by intraflagellar transport (IFT). IFT is a bi-directional movement of protein complexes along the axoneme (Silverman *et al.*, 2009). The lack of protein synthesis machinery in cilia/flagella leads to a problem of targeting proteins to the ciliary base and specifically importing them into the ciliary compartment (Taschner *et al.*, 2012). IFT uses kinesin anterograde molecular motor(s) to mobilize ciliary cargo within cilia and cytoplasmic Dynein 1b to recycle components back to the base (Silverman *et al.*, 2009).

Based in studies performed by Inaba *et al* in rainbow trout and salmon sperm, and the model of spermatozoa motility acquisition proposed by Fardilha *et al*. (Section I.6.1), TCTEX1D4 seems to be a substrate for PPP1CC2 and its phosphorylation state may determine if spermatozoa is capable of movement (Korrodi-Gregório, 2012). In midpiece, where mitochondria are concentrated, TCTEX1D4 is highly enriched and therefore may be involved in energy production necessary for flagellar motility. Concerning the head, TCTEX1D4-PPP1CC2 complex might have a role in the acrosome reaction and consequently in fertilization, since co-localization of both proteins is stronger in the acrosome region (Korrodi-Gregório, 2012). In Figure 7 is shown a representation of TCTEX1D4, PPP1CC2 and possible TCTEX1D4-PPP1CC2 complexes localization in spermatozoa and their putative functions.

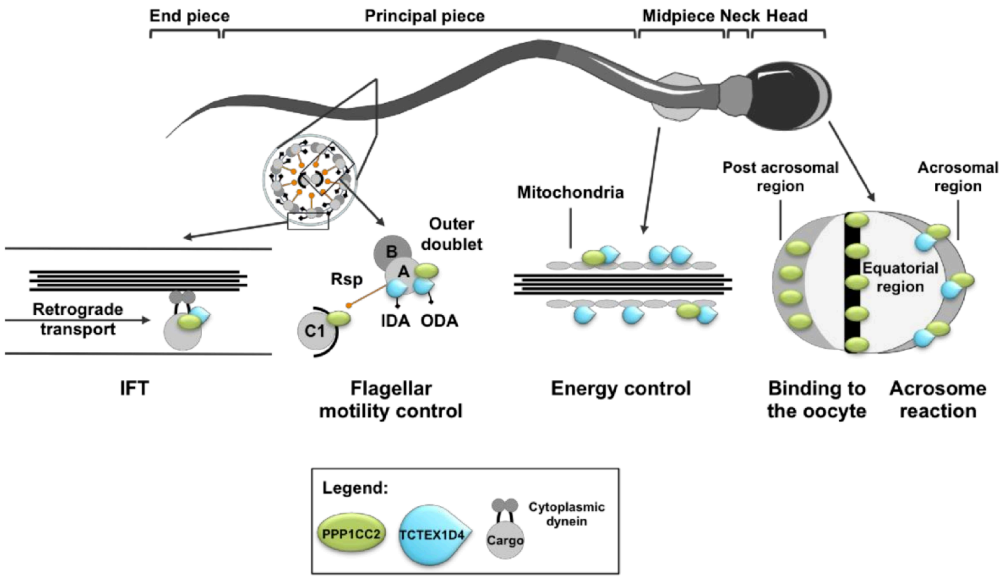


Figure 7. Representation of TCTEX1D4, PPP1CC2 and possible TCTEX1D4-PPP1CC2 complexes localization in spermatozoa. TCTEX1D4 (in blue) and PPP1CC2 (in green) are present in head and tail of human spermatozoa. In the head TCTEX1D4-PPP1CC2 complex may have a role in the acrosome reaction and along the tail can mediate flagellar motility and/or intraflagellar transport (IFT). It may also have a role in energy control, since it is present in midpiece (houses the mitochondria). Rsp, radial spoke; C1 central pair microtubule 1; B, tubule B; A, tubule A; IDA, inner dynein arm; ODA, outer dynein arm (Korrodi-Gregório, 2012).

Although the knowledge about TCTEX1D4 contribution to male reproduction is starting to be uncovered much work is still ahead of us. In a clinical point of view, understanding the control of spermatogenesis and spermatozoa motility can uncover therapeutic targets for treatment of male infertility or even for male contraception.

I.8. Yeast Two Hybrid System: Unveiling protein-protein interactions

The Yeast Two Hybrid System (YTH) was developed in the end of the 80´s. It was one of the first techniques that allowed the detection of protein-protein interactions *in vivo*, in the yeast *Saccharomyces cerevisiae*. Previously, the detection of protein-protein interactions was done using *in vitro* techniques, such as, immunoprecipitation (biochemical approaches) (Fields *et al.*, 1989). The principle of the YTH system technology relies in two properties of eukaryotic transcription factors (Colas *et al.*, 1998; Bruckner *et al.*, 2009):

- Transcription of a gene is only possible in presence of the DNA binding domain (BD) of a transcription factor and the activation domain (AD) of a transcription factor. The binding domain interacts with upstream activation domain (UAS).
- The two domains only need to be in proximity to each other (covalent bond is not necessary) and therefore do not need to be in the same polypeptide.

Inspired by these two characteristics, Fields and Song proposed a simple method for protein-protein interaction detection. The basic idea was to fuse two proteins of interest, X and Y, to the BD and AD, respectively, of one transcription factor. Gal4, a transcription factor in yeast that activates transcription of beta galactosidase when galactose is present in the surrounding environment, was the chosen one. If protein X and Y interacted, Gal4 was restored and became functional leading to the recruitment of RNA polymerase II. As consequence, beta galactosidase was expressed and galactose degraded. If X gal is present in the medium a chromogenic substrate is produced and it is possible to identify positive clones (turn blue) (Luban *et al.*, 1995; Bruckner *et al.*, 2009)

Using the same principle, a genome wide screen for interactors of a particular protein can be performed. A known protein, bait, is introduced into a vector, which contains the gene for the transcription factor BD. Then, the vector is transformed into a specific yeast strain. A cDNA library, containing prey cDNAs, is introduced into a vector, which contains the gene for transcription factor AD and introduced into a yeast strain that is capable of mating with the bait yeast strain. After mating, any prey that interacts with the bait leads to the expression of the reporter gene and is identified as an interacting protein (Luban *et al.*, 1995; Colas *et al.*, 1998; Bruckner *et al.*, 2009). Nowadays, more than one reporter gene is used, normally combining a colorimetric reaction and auxotrophic markers that restrict yeast growth in a minimal media. These, decreases the percentage of false positives (for example due to non-specific interactions) because it requires

a more solid transcription (Bruckner *et al.*, 2009). It should be noted that detection of a positive interaction must be reconfirmed by other methods. (Luban *et al.*, 1995). In Figure 8 is an illustrative diagram of the YTH system principle.

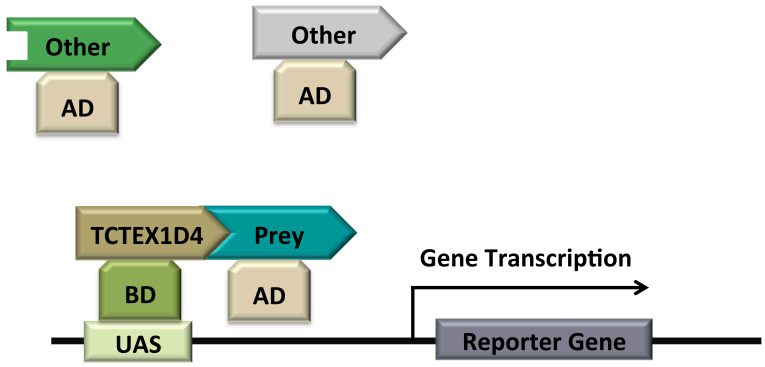


Figure 8. Illustrative diagram of yeast two-hybrid system principle. TCTEX1D4 is fused with transcriptional factor binding domain (BD) that binds to the upstream activation domain (UAS). Protein derived from testis cDNA library is fused with the transcription factor AD. If TCTEX1D4 and a given library protein interact, the two domains are brought together and transcription of a reporter gene occurs.

Although YTH system is a well established approach for protein-protein interaction, there are a few constrains that may occur.

- The proteins used, bait and prey, must be expressed as stable fusion proteins.
- The fusion proteins must be transported to the nucleus (where the activation of transcription occurs).
- The protein of interest fused with the BD may be capable of self activate transcription. In alternative, smaller individual domains of the protein can be used. However, this approach can itself raise another problem. Certain domains may be "hidden" in the wild type protein and therefore don´t interact with the protein of interest in normal conditions (Luban *et al.*, 1995).
- Many proteins need post transcriptional modifications to interact (glycosylation, phosphorylation, etc.). Yeast may not perform such modification properly (Fields *et al.*, 1994) and consequently false negatives can occur.
- The interactions are detected in yeast and some proteins when expressed in these organisms may be toxic and therefore false negatives.

Nevertheless, the YTH system was used to generate most of the published protein-protein interaction data. For example, more than 5,600 protein interactions have been reported for yeast

and 6,000 for humans using YTH system. Approximately half of the interaction data available on databases such as IntAct and MINT are produced by YTH system approach (Bruckner *et al.*, 2009). Nowadays, YTH system is the elected approach to perform a large scale mapping of protein-protein interaction and ultimately, in the future, build a proteome wide binary protein-protein interaction maps (Koegl *et al.*, 2007).

II. AIMS

In order to identify TCTEX1D4-PPP1CC2 complex functions in spermatogenesis, spermatozoa motility and fertilization, TCTEX1D4 partners were identified. Thus, an YTH screen of a human testis library using TCTEX1D4 as bait was previously performed. Using this approach we will be able to identify TCTEX1D4 interactome and at the same time shed light on putative trimeric complexes formed with PPP1. By identifying TCTEX1D4 interacting proteins, new functions will be revealed and therefore we will unveil the importance of TCTEX1D4 in male reproduction. Ultimately, finding new therapeutic targets for male infertility diagnosis or therapeutics will be possible. So the specific aims of the master thesis are:

1. To identify the TCTEX1D4 interacting proteins fused to the GAL4-AD, by DNA sequencing of the testis library cDNA inserts,
2. To analyze the interacting proteins identified, by an in *silico* approach (protein identification, chromosomal localization of the gene, isoforms, expression pattern, presence of a putative PPP1 binding motif, cellular functions).
3. To organize the TCTEX1D4 interactome
4. To describe putative functions of the TCTEX1D4 complexes.

III. METHODS AND MATERIALS

III.1. Library construction and selection of the positive clones

All solution compositions are in Appendix I.

In order to identify TCTEX1D4 interacting proteins an yeast two hybrid system (YTH) was performed. The YTH used was the Matchmaker Gal4 Two-Hybrid System 2 (Clontech, Saint Germain-en-Laye, France). To produce Tctex1d4^{pAS2-1}, pAS2-1 was digested with NcoI-SalI and Tctex1d4 was inserted into the vector. Then the Tctex1d4^{pAS2-1} was transformed into AH109 yeast strain. Yeast transformations were performed using a standard lithium acetate method (Matchmaker Yeast Protocols Handbook, Clontech, Saint Germain-en-Laye, France). Yeast strain Y187 expressing a human testis cDNA library cloned in pACT-2 was used as mating partner of AH109. Positive colonies were obtained on selective media (containing X-α-GAL to check expression of MEL1 a reporter gene) (Fardilha *et al.*, 2011b). This work was previously performed by Luis Korrodi-Gregório, in Signal Transduction Laboratory.

III.2.Plasmid isolation from yeast

A single yeast colony was grown in 3mL supplement quadruple dropout medium (SD/QDO) at 30ºC and 180 rotations per minute (rpm) (2-3days). Then, they were pellet for 3min at room temperature (RT) and resuspended in 100μL of STET solution. About 300μL of 0,5mm acid-washed beads (Sigma-Aldrich Química, S.A., Sintra) were added and vortexed on high speed for 6-8min. Again 100μL of STET were added and the tubes were boiled for 3min and chilled on ice immediately. After, the tubes were centrifuged for 10min at 4ºC, maximum speed and the supernatant was transferred into a new tube, already with 500μL of ammonium acetate 7.5M. The mix was briefly homogenized and incubated overnight (ON) at -20ºC. Then, the tubes were centrifuged for 20min at 4ºC, maximum speed and 500μL of the supernatant was transferred to a new tube with 800μL of ice-cold ethanol 100% (MERCK, Darmstadt, Germany). The mix was incubated for 2-3hrs at -20ºC and spin down for 10min at 4ºC, 1.500g. After removing the supernatant, 800μL of ethanol 70% was added (MERCK, Darmstadt, Germany) and let it stand for 5min at 20ºC. The tubes were centrifuged for 5min at 4ºC, maximum speed and the pellet was air dried at until no traces of ethanol was left. Finally, the pellet was resuspended in 15μL of sterile water with RNase (Sigma-Aldrich Química, S.A., Sintra) (20μg/mL).

III.3. Bacterial transformation

III.3.1. Preparation of E. coli XL1-Blue competent cells

A pre-culture of *E. coli* XL1-Blue was grown ON with shaking at 37ºC in 10mL of SOB medium. In 50mL of SOB medium was inoculated 1mL of the pre-culture and incubated for 1-2hrs at 37ºC with shaking (180rpm) until OD_{550mm}=0,3. The culture was chilled on ice for 15min and centrifuged for 5min at 4ºC, 1.500g. Then the supernatant was removed and the pellet was resuspended in 15mL of solution I. After 15min on ice, the mix was centrifuged for 15min at 4ºC, 1.500g. The supernatant was removed and the cell pellet resuspended in 3mL of solution II. The cells were divided in 100μL aliquots, immediately frozen and stored at -80ºC.

III.3.2. Bacterial transformation with plasmid DNA

E. coli XL1-Blue competent cells were thawed on ice, 100μL were added to the plasmid DNA and gently swirled. The cells were incubated on ice for 15-20min. Then, the thermic shock was performed, by exposing the cells to 42ºC for 90sec and rapidly cooled by incubating for 25-30min on ice. After, 900 μL of SOC was added and incubated for 45min at 37ºC with shaking at 180rpm. The cells were pelleted for 2min and the supernatant discarded, with the exception of around 100μL in which the cells were resuspended. Finally the cells were spread on LB/Ampicillin (50μg/mL) agar plates and incubated at 37ºC for 16-18hrs until colonies appear.

III.4. Plasmid analysis

In order to screen for the recombinant plasmid in the transformants, the plasmid DNA was extracted from 3 isolated colonies using an alkaline lysis approach and digested with the restriction endonuclease, *HindIII*. When separated by agarose gel electrophoresis, the fragment pattern for pACT-2 + library allows the differentiation of the colonies resulting from transformation of the bait vector. The plasmids that generated DNA fragments characteristic of the pACT-2 + library insert were further analyzed by sequencing.

III.4.1. Plasmid DNA extraction from bacteria – Alkaline lysis "mini prep"

A single bacterial colony was transferred into 3mL of LB/Ampicillin (50μg/mL) and incubated for 16-18hrs at 37ºC with shaking (180rpm). Then, 1.5mL of the culture was transferred into a microtube and centrifuged for 1min at RT. The supernatant was discarded and the cell pellet was resuspended in 100μL of ice cold Resuspension solution. Then, 200μL of Lysis solution was added

and the mix was inverted gently several times. Quickly, 150μL of ice cooled Neutralization solution was added and the tubes were, again, inverted several times. The mix was incubated on ice for 5min and centrifuged for 10min at 4ºC, maximum speed and the supernatant was transferred to a new microtube with 2 volumes of ice-cold ethanol 100%. After briefly vortexing, the DNA was incubated for 2-3hrs at -20ºC. Then, the microtubes were centrifuged for 10min at 4ºC, maximum speed and the supernatant was removed. The pellet was resuspended in 800μL of ice-cold ethanol 70%. Following a centrifugation for 5min at 4ºC, maximum speed, the pellet was air dried and resuspended in 20μL of sterile RNase solution (20μL/mL).

III.4.2. Restriction fragment analysis of DNA

The plasmidic DNAs were analyzed through the digestion with *HindIII* (New England Biolabs, Herts, UK). For the digestion the following components were used:

VOLUMES (μL)	10 TUBES	15 TUBES	20 TUBES
Final Volume	120	200	250
H_2O	45	77	96
Buffer2 10x	12	20	25
HindIII	3	3	4
Plasmidic DNA	60	100	125

The mixture was incubated at 37ºC ON.

III.4.3. Electrophoretic analysis of plasmidic DNA

To visualize the DNA fragments an agarose gel electrophoresis was prepared and the DNA fragments were separated by molecular size. A 1% agarose (Invitrogen, Life Technologies S.A., Madrid, Spain) gel was prepared in TAE 1x and ethidium bromide (Sigma-Aldrich Química, S.A., Sintra) was added to a final concentration of 0.5μg/mL. When the mix was about 60ºC it was poured into the appropriate mold and the comb was placed. The gel was allowed to polymerize for 30min. The samples and 1kb plus marker (Invitrogen, Life Technologies S.A., Madrid, Spain) were mixed with 6x loading buffer. Once the gel was solid it was placed into the tank field with TAE 1x and the comb removed carefully. The samples and the marker were loaded into the wells and the gel was run at 100V. When the dye had migrated the appropriate distance, the gel was

examined under a UV light, using Alpha Imager HP (Alpha Innotech, California USA), and the band pattern was analyzed.

III.4.4. DNA sequencing

Since the DNA samples were obtained by Alkaline lysis "mini prep", they were purified by one of two methods: A QIAquick spin column (QIAGEN, Dusseldorf, Germany), or an ethanol precipitation.

QIAquick Spin Column

To the plasmid DNA it was added 5 volumes of buffer PBI to 1 volume of DNA solution and mixed by vortexing. If the color of the mix turned orange or violet, 10μL of 3M sodium acetate was added. The mix was applied to the QIAquick column and centrifuged for 1min at 10.000g, RT to bind the DNA. The flow-through was discarded and the column was placed back into the same tube. After, 750μL of buffer PE was added to the column and centrifuged for 1min at 10.000g and RT. Again, the flow-through was discarded and the column was placed in the same microtube and centrifuged for an additional minute. Then, the column was placed in a new 1.5mL microtube and placed for about 5min at 37ºC to unsure that all buffer PE was removed. Finally, 50μL of buffer elution buffer (EB) was added, allowed to stand for 1min and centrifuged for 1min at 10.000g, RT. The DNA was stored at -20ºC.

Ethanol Precipitation

To the plasmid DNA 1/10 volume of sodium acetate and 2.5 volumes of ice-cold ethanol 100% were added and mixed by vortexing. The mixes were incubated ON at -20ºC. Then, they were centrifuged for 20min, 4ºC at maximum speed, and the supernatant was discarded. After, 750μL of iced cold ethanol 70% was added and the tubes were kept at -20ºC, for 5min. The mixes were centrifuged for 5min, 4ºC at maximum speed, the supernatant was rejected and the pellet dried at 37ºC to eliminate any traces of ethanol. The pelleted DNA was resuspended in 30-50μL of sterile water and stored at -20ºC.

Polymerase Chain Reaction (PCR)

For the sequencing PCR reaction the following components were mix in a 0,2mL microtube:

- 500ηg of plasmidic DNA

- 4μL Ready Reaction Mix (dye terminators; deoxynucleoside triphosphatases; AmpliTaq DNA polymerase, FS; *Thermus thermophilus* pyrophosphatase; magnesium chloride and buffer) (Portugal Applied Biosystems, Porto, Portugal).
- 10pmol primer GAL4-AD (Clontech, Saint Germain-en-Laye, France)
- H_2O to a final volume of 20μL

The reaction was mixed by tapping and then spin down for a few seconds. The PCR was performed with the following conditions in a BioRad MyCycler Thermal Cycler (BioRad Portugal, Lisboa, Portugal):

96ºC 1min	
96ºC 30sec	25 cycles
42ºC 15sec	
60ºC 4min	

Ethanol Precipitation for Sequencing

After PCR, the DNA was purified by an ethanol precipitation method. The mixes were placed in a new 1.5mL microtube and 1/10 volume of sodium acetate and 2.5 volumes of RT ethanol were added. After vortexing, the mixes were incubated for 20min at RT and centrifuged for 20min at maximum speed, RT. The supernatant was discarded and 250μL of ethanol 70% was added and mixed by vortexing. Again the mixes were centrifuged for 5min at maximum speed, RT and the supernatant was discarded. After drying the pellet, the DNA was ready to be sequenced in a Automated DNA sequencer (ABIPRISM 310, Portugal Applied Biosystems, Porto, Portugal).

III.5. Sequence analysis – Bioinformatics

The nucleotide sequence obtained from each clone was visualized and analyzed using Finch TV™ software (http://www.geospiza.com/Products/finchtv.shtml) that enables the visualization of chromatograms and a direct BLAST search. The obtained sequences were compared with GenBank Databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of human nucleotide sequences enabling the identification of the TCTEX1D4 interacting proteins.

The structural and functional characterization of TCTEX1D4 interacting proteins was possible using databases, such as, UniProt, NCBI, Human Protein Reference Database (HPRD), ScanProsite (Swiss-Prot). Protein networks where construct using the software Cytoscape™, an open source bioinformatics software platform developed by a collaboration between Agilent technologies;

University of Toronto; University of San Francisco, Institut Pasteur; Memorial Sloan-Kettering Cancer Center; University of California, San Diego and Institute for Systems Biology. With Cytoscape™ is possible to visualize molecular interaction networks and biological pathways (http://www.cytoscape.org/).

IV. RESULTS AND DISCUSSION

IV.1. Identification of the Yeast Two Hybrid positive clones

From a total of 2,59x10^6 screened clones, the YTH system produced 494 positive clones. From these, 86 clones were identified corresponding to 41 different proteins, 1 open reading frame and 3 genomic contig. The analyzed clones were partially sequenced and identified by nucleotide search using Genebank database (http://blast.ncbi.nlm.nih.gov/).

In order to identify the insert present in a particular positive clone, the DNA was first isolated. However, a single yeast clone can incorporate more than one plasmid from the library and the bait plasmid. Therefore, to obtain single plasmids, the DNA was first transferred to bacteria *E. coli* XL1-Blue by transformation and analyzed by restriction digestion with the endonuclease *HindIII*. The restriction fragments were resolved by agarose gel electrophoresis and since the fragment patterns obtained for library insert and bait plasmids are completely different, it is possible to distinguish between them. Figure 9 represents an example of the fragments pattern obtained. When *HindIII* cuts the pAS2-1-TCTEX1D4 the obtained band patter is the one shown in lane L4; When *HindIII* cuts a library plasmid there is always a 7,4Kb band and the other(s) band(s) obtained vary with the insert (lane L2 and L3).

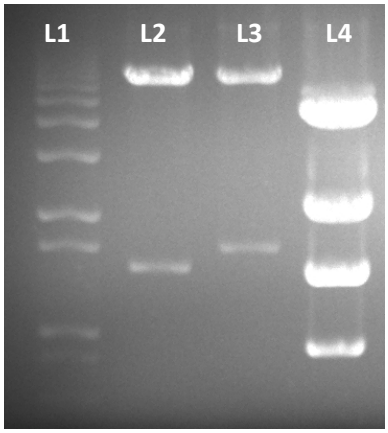


Figure 9. Fragment pattern produced by *HindIII* endonuclease digestion. L1, 1Kb+ DNA ladder marker. L2 and L3, pattern obtained when *HindIII* cuts pACT2-library inserts [(7,4Kb + (0,8 + insert)]. L4, pattern obtained when *HindIII* cuts pAS2-1-TCTEX1D4 [(4,6Kb+2,2Kb+(1,4Kb+TCTEX1D4)+0,9Kb)].

All positive clones underwent the same method allowing the identification of clones carrying cDNA library plasmids. After sequencing, the nucleotide sequence obtained for each clone (Figure 10) was compared against the GenBank Database of human nucleotide sequences by BLAST search, (Figure 11) and the identity of the positive clone retrieved (Figure 12).

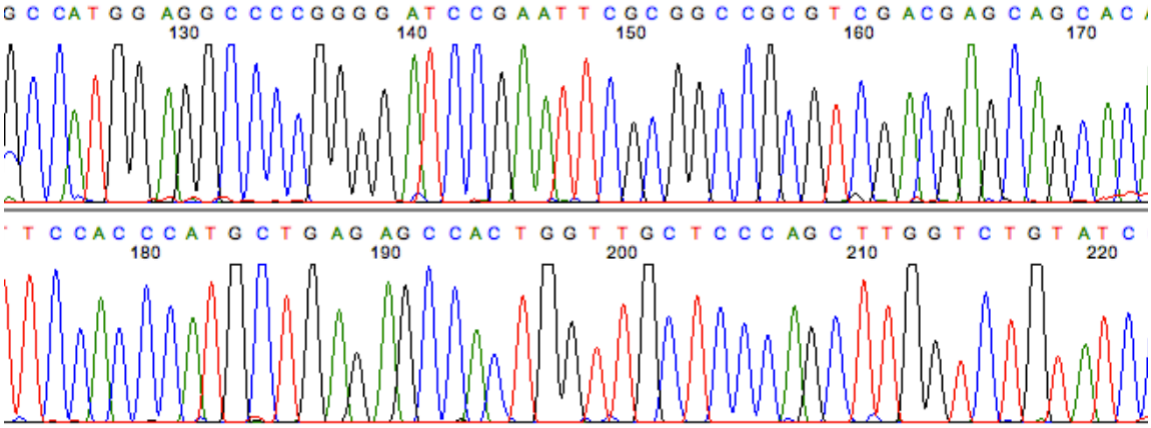


Figure 10. Partial sequence of a positive clone. The chromatogram was visualized using the FinchTV software.

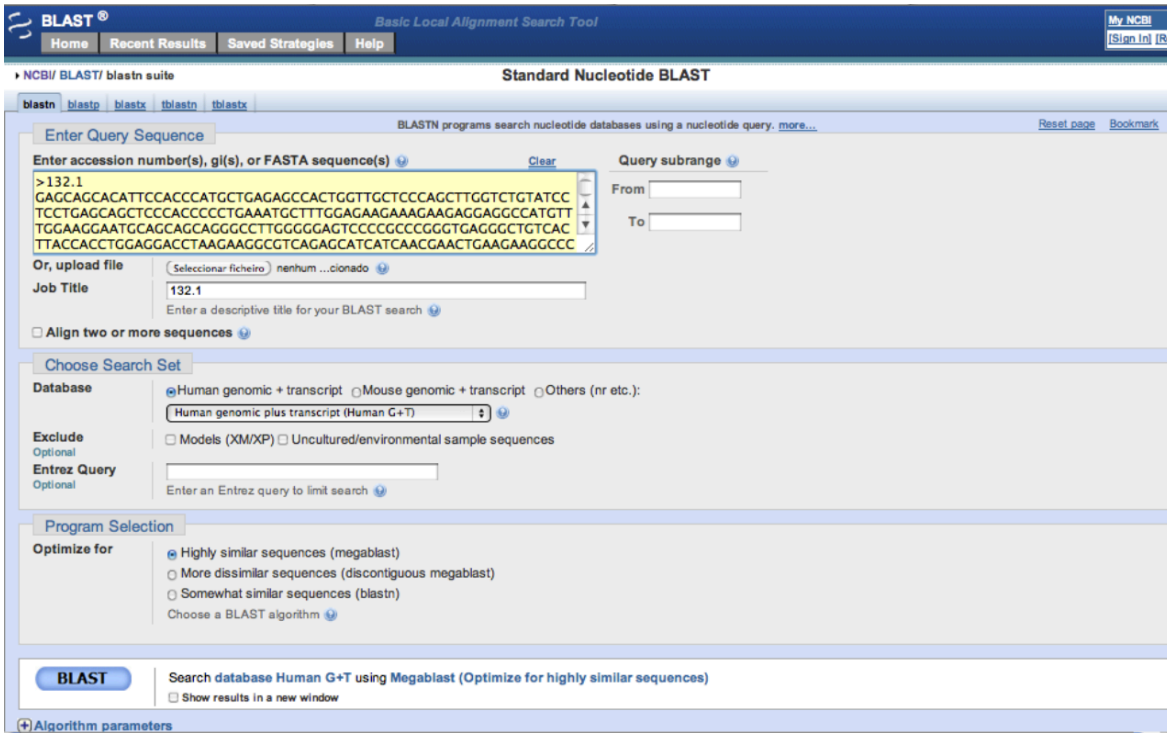


Figure 11. BLAST window. The query sequence was introduced in the blast window and a database search was undertaken.

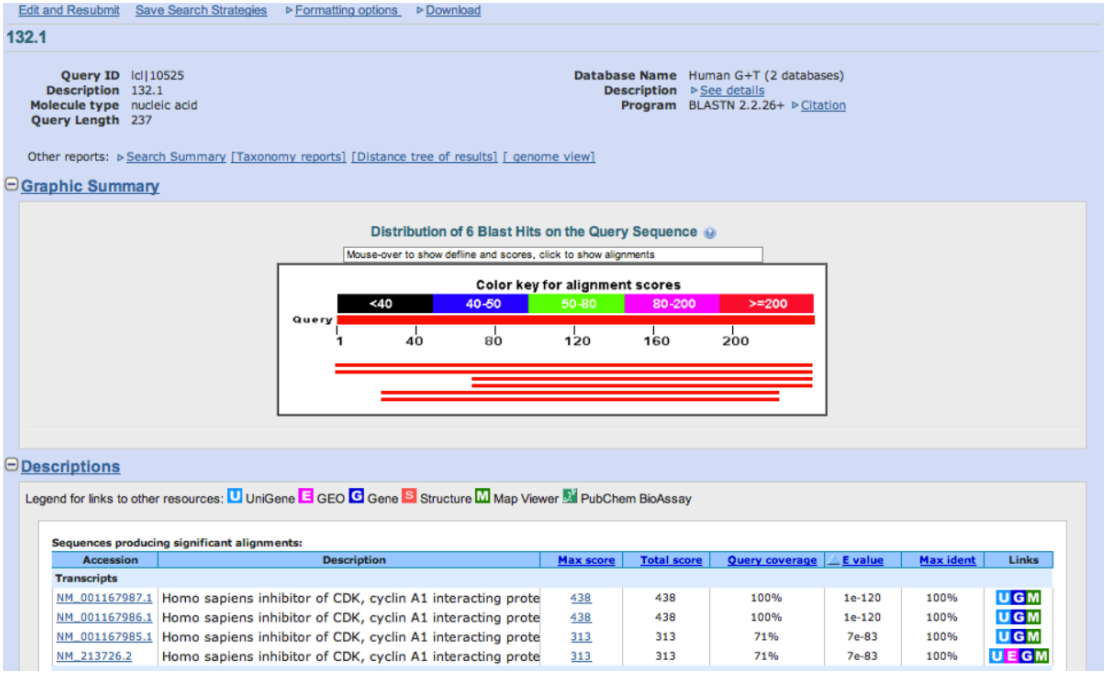


Figure 12. Blast result. The alignment shows that Inhibitor of CDK, cyclin A1 interacting protein 1 aligns with the insert cDNA present in positive clone T132.1.

IV.2. Analysis of the positive clones

To better characterize all interacting proteins, the full-length sequence was retrieved from the databases and PPP1 binding motifs search was performed in all positive clones, using ScanProsite tool (http://prosite.expasy.org/scanprosite/). The presence of PPP1BM may indicate a putative interaction with PPP1. However these binding motifs are common to all PPP1 and therefore are not unique to PPP1CC2. Since PPP1CC2 colocalizes with TCTEX1D4 and is involved in spermatozoa motility (Vijayaraghavan *et al.*, 1996; Korrodi-Gregório, 2012), the presence of PPP1 binding motifs in TCTEX1D4 interacting proteins can be a clue to the involvement of such proteins in spermatozoa motility together with TCTEX1D4. In Table 4 are listed all PPP1 binding motif sequences searched.

Table 4. Putative PPP1 binding motifs. All identified proteins were analyzed to find PPP1 binding motifs. X(0,1) is any aa, present or absent; [P] represents any aa except Proline (P); {FIMYDP} represents any aa except F/I/M/Y/D/P.

PPP1 BINDING MOTIF	
RVxF	[RK]-X(0,1)-[VI]-{P}-[FW]
	[HKR]-[ACHKMNQRSTV]-V-[CHKNQRST]-[FW]
	[KRL]-[KRSTAMVHNQ]-[VI]-{FIMYDP}-[FW]

PPP1 BINDING MOTIF	
MyPhone	R-X-X-Q-[VIL]-[KR]-X-[YW]
PPP1R2 degenerate motif	R-[KR]-X-H-Y
	K-S-Q-K-W
SILK motif	K(0,1)-[GS]-I-L-K
Other motifs	F-X-X-[RK]-X-[RK]
	R-A-R-A
	R-N-Y-F

Table 5 lists all 44 TCTEX1D4 interacting proteins, as well as, the number of positive clones, UniProt Identification number, chromosomal localization of the responding gene, subcellular localization, isoforms and presence of putative PPP1 binding motifs. Besides messages that code for proteins, were also identified several open reading frames and genomic contigs. Open reading frames (ORFs) identified are the part of the gene that begins with a start codon and ends with stop codon. However, it is not known if the ORF is transduced and translated (Griffiths *et al.*, 2004). In databases Genomic contig was introduced by Staden in 1980 due to the high quantity of information produced by shotgun sequencing. Genomic contig is a set of DNA readings, produced by shotgun sequencing, that are related to one another by overlap of their sequences. The readings in a contig can be ordered to form a continuous consensus sequence (Staden, 1980).

Table 5. TCTEX1D4 interacting proteins – the human testis TCTEX1D4 interactome. Gene locus and subcellular localization were retrieved from UniProt, NCBI and HPRD databases. PPP1BM were obtained by full sequence scan using ScanProsite tool (Swiss-Prot). The described function was retrieved from Uniprot and NCBI.

Proteins	No. Clones	Uniprot ID	Gene Locus	Isoforms Specificity	Subcellular Localization	PPP1BM	Function
ACTN1	1	P12814	14q22-q24	All 3 isoforms	Cell membrane Cytoplasm Cytoskeleton	RVGW	Alpha actinin is an actin-binding protein with multiple roles in different cell types. In non-muscle cells, is involved in actin binding to the membrane.
ATXN7L1	1	Q9ULK2	7q22.3	Isoform 1 and 3	-	-	Ataxin-7-like protein 1
CCDC89	5	Q8N998	11q14.1	Unique isoform	-	-	Coiled-coil domain-containing protein 89
CRISP2	4	P16562	6p12.3	All 5 isoforms	Secreted	-	Cystein-Rich Secretory protein 2 may regulate some ion channels activity and thereby regulate calcium fluxes during sperm capacitation. It is present in the acrosome
CTSB	1	P07858	8p22	Unique isoform	Lysosome	RVMF	Cathepsin B is an amyloid precursor protein (APP) secretase. Incomplete proteolytic processing of APP has been suggested to be a causative factor in Alzheimer disease.
CTSL1	1	P07711	9q21.33	Isoform 1	Lysosome	RSVDW	Cathepsin L1 is a lysosomal cysteine proteinase that plays a major role in intracellular protein catabolism.
FBLN1	1	P23142	22q13.31	Isoform C	Secreted	-	Fibulin 1 is a secreted glycoprotein that becomes incorporated into a fibrillar extracellular matrix. It mediates platelet adhesion via binding to fibrinogen.

Proteins	No. Clones	Uniprot ID	Gene Locus	Isoforms Specificity	Subcellular Localization	PPP1BM	Function
FLOT1	1	O75955	6p21.3	Unique isoform	Cell membrane Endosome (membrane)	RARA	Flotilin-1 encodes a caveolae-associated, integral membrane protein. Caveolae are small domains on the inner cell membrane involved in vesicular trafficking and signal transduction.
FNDC8	3	Q8TC99	17q12	Unique isoform	-	-	Gem-associated protein 4
GALNTL2	1	Q8N3T1	3p25.1	Unique isoform	Golgi apparatus (membrane)	KEIHF	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 2 catalyzes the initial reaction in O-linked oligosaccharide biosynthesis.
GARS	1	P41250	7p15	Unique isoform	Cytoplasm Mitochondria	-	Glycyl-tRNA synthetase is one of the aminoacyl-tRNA synthetases that charge tRNAs with their cognate amino acids.
GEMIN4	1	P57678	17p13	Unique isoform	Cytoplasm Nucleus Spliceosome	LSVLF	GEMIN4 is a component of the survival of motor neurons protein
GNB2L1	1	P63244	5q35.3	Unique isoform	Cell membrane Cell projection Cytoplasm Cytoskeleton Nucleus (membrane)	LSVAF	Guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1 is Involved in the recruitment, assembly and/or regulation of a variety of signaling molecules.
H-IDHB	1	O43837	20p13	All 3 isoforms	Mitochondria	KIKF	Isocitrate dehydrogenases catalyze the oxidative decarboxylation of isocitrate to 2-oxoglutarate.

Proteins	No. Clones	Uniprot ID	Gene Locus	Isoforms Specificity	Subcellular Localization	PPP1BM	Function
HNRNPF	1	P52597	10q11.21	Unique isoform	Nucleus Spliceosome	FVVKLR	Heterogeneous nuclear ribonucleoprotein F is associated with pre-mRNAs in the nucleus and regulates alternative splicing, polyadenylation, and other aspects of mRNA metabolism and transport.
IFT88	2	Q13099	13q12.1	All 2 isoforms	Cell projection Cilium Cytoplasm Cytoskeleton	KAIKF GILK	Intraflagellar transport 88 homolog (*Chlamydomonas*) is a member of the tetratrico peptide repeat (TPR) family
INCA1	19	Q0VD86	17p13.2	All 2 isoforms	Nucleus	-	Inhibitor of CDK interacting with cyclin A. Involved in regulation of transcription and mRNa processing
LRRC71	1	Q8N4P6	1q23.1	Unique isoform	-	KYVFF	Leucine-rich repeat-containing protein 71
MAN2B1	2	O00754	19cen-q13.1	Isoform 1	Lysosome	RKVNW RVAW	Mannosidase, alpha, class 2B, member 1 is an enzyme necessary for the catabolism of N-linked carbohydrates released during glycoprotein.
OSGEP	4	Q9NPF4	14q11.2	Unique isoform	-	-	O-sialoglycoprotein endopeptidases specifically cleave the polypeptide backbone of membrane glycoproteins that contain clusters of O-linked sialoglycans
PDXP	2	Q96GD0	22cen-q12.3	Unique isoform	Cell membrane Cytoplasm Cytoskeleton	-	Pyridoxal phosphatase dephosphorylates pyridoxal 5-prime-phosphate (PLP) the active form of vitamin B6.
QRICH1	1	Q2TAL8	3p21.31	All 2 isoforms	-	-	Glutamine-rich protein 1, present a caspase activation recruitment domain which suggests the involved in

Proteins	No. Clones	Uniprot ID	Gene Locus	Isoforms Specificity	Subcellular Localization	PPP1BM	Function
							apoptosis and inflammation
RANBP9	1	Q96S59	6p23	Unique isoform	Cytoplasm Nucleus	RMIHF	RANBP9 is essential for the translocation of RNA and proteins through the nuclear pore complex.
RBM4B	1	Q9BQ04	11q13	Unique isoform	Nucleus	-	RNA binding motif protein 4B may play a role in alternative splice site selection.
SECISBP2	2	Q96T21	9q22.2	Unique isoform	Nucleus	LNVAW	SECIS binding protein 2 is a nuclear protein that incorporates selenocysteine into proteins necessary for mRNA processing.
SMURF1	1	Q9HCE7	7q22.1	All 3 isoforms	Cytoplasm Cell Membrane	RIYF LMVKF	SMAD specific E3 ubiquitin protein ligase 1 is a ubiquitin ligase that is specific for receptor-regulated SMAD proteins in the bone morphogenetic protein pathway.
STAM2	1	O75886	2q23.3	Unique isoform	Cytoplasm Endosome (membrane)	LMVEW	Signal transducing adaptor molecule 2 is an adaptor protein involved in the downstream signaling of cytokine receptors.
TCTEX1D2	1	Q8WW35	3q29		-	FQQRFR	TCTEX1D2 appears to be important in the assemble of dyneins
TECR	1	Q9NZ01	19p13.12	Unique isoform	Endoplasmic reticulum (membrane)	–	Trans-2,3-enoyl-CoA reductase is a multi-pass membrane protein that resides in the endoplasmic reticulum. This protein catalyzes the final step of the elongation of microsomal long and very long chain fatty acid.
TIM50	1	Q330K1	19q13.2	Unique isoform	Mitochondria inner membrane	-	Translocase of inner mitochondrial membrane 50 homolog is a subunit of the TIM23 complex that links protein translocation across the outer and inner mitochondrial membranes

Proteins	No. Clones	Uniprot ID	Gene Locus	Isoforms Specificity	Subcellular Localization	PPP1BM	Function
TSC21	1	Q96LM6	2p11.2	Unique isoform	Nucleus	-	Testis-specific conserved protein of 21 kDa
TTC28	2	Q96AY4	22q12.1	All 2 isoforms	-	FVEKVR	Tetratricopeptide repeat protein 28
UBE2D2	4	P62837	5q31.2	Isoform 2	Cytoplasm	KVAF LTIHF	Ubiquitin-conjugating enzyme E2D 2 modifies proteins with ubiquitin being an important cellular mechanism for targeting abnormal or short-lived proteins for degradation.
UQCRC1	1	P31930	3p21.3	Unique isoform	Mitochondria inner membrane	RDVVF	*Homo sapiens* ubiquinol-cytochrome c reductase core protein I is a component of the ubiquinol-cytochrome c reductase complex, which is part of the mitochondrial respiratory chain.
USP33	1	Q8TEY7	1p31.1	All 3 isoforms	Cytoplasm	-	Ubiquitin specific peptidase 33 is involved in various deubiquitinating processes such as cellular migration and beta-2 adrenergic receptor/ADRB2 recycling.
WDR73	2	Q6P4I2	15q25.2	Unique isoform	-	LRVTW	WD repeat-containing protein 73
ZIM2	1	Q9NZV7	19q13.4	Unique isoform	Nucleus	-	Zinc finger, imprinted 2 may be involved in transcriptional regulation
ZNF335	2	Q9H4Z2	20q13.12	Unique isoform	Nucleus	-	Zinc finger protein 335 enhances transcriptional activation by ligand-bound nuclear hormone receptors
ZNF562	1	Q6V9R5	19p13.2	All 3 isoforms	Nucleus	FEEKTK	Zinc finger protein 562 may be involved in transcriptional regulation.
ZNF638	2	Q14966	2p13.1	All 2 isoforms	Nucleus	FMAKQR	Zinc finger protein 638 is a nucleoplasmic protein. It binds cystidine-rich in DNA and it is associated with packaging,

PROTEINS	NO. CLONES	UNIPROT ID	GENE LOCUS	ISOFORMS SPECIFICITY	SUBCELLULAR LOCALIZATION	PPP1BM	FUNCTION
						FISRYR	transfering or processing transcripts.
Chromosome 11 genomic contig	1	_	11	_	_	_	-
Chromosome 11 genomic contig, GRCh37.p5 Primary Assembly	2	_	11	_	_	_	-
Chromosome 12 open reading frame 63	1	_	12	_	_	KEVHF	-
Chromosome 19 genomic contig, GRCh37.p5	1	_	19		_	_	-

The differences in the number of positive clones can hint the level of expression of such proteins in human testis. For example, the protein inhibitor of CDK, Cyclin A1 interacting protein (INCA1), was identified 19 times denoting a high level of expression of this protein in human testis. To rapidly and easily exclude all the clones transformed with an abundant cDNA insert, such as INCA1, a yeast colony hybridization can be performed. In these technique yeast are grown in a selective media and lifted onto a nylon membrane and the cell wall is destroyed, exposing the DNA. After radio labeling the DNA of interest (abundant DNA), it is incubated with the exposed DNA. Identical DNA hybridize and after exposing to a film it is possible to assess which transformants have the same DNA and thus exclude them from the single sequencing procedure (Clontech, 2009).

IV.3. TCTEX1D4 interacting proteins tissue distribution

To identify testis specific or enriched proteins and therefore specify possible complexes unique to testis, the expression pattern of all TCTEX1D4 interacting proteins in *Homo sapiens* were retrieve from NCBI (UniGene EST profile). Besides TCTEX1D4 interacting proteins, pattern of expression of TCTEX1D4, PPP1CC and protamine 2 (PRM2) were also retrieved. PRM2 substitutes the histones in the chromatin of spermatids in late spermatogenesis and compact sperm DNA into a highly condensed complex (Reid *et al.*, 2011). PRM2 is a known and well-characterized testis specific protein and therefore the pattern of expression is testis specific. The information presented is an approximation of the gene expression patterns inferred from expressed sequence tags of normalized libraries and hence the values may not be a true indicator of gene activity. Also some of the tissue expression patterns are not presented here although all tissues were considered in the analysis. In Table 6 are presented the tissue/body sites of TCTEX1D4, PPP1CC and PRM2 expression pattern and in Table 7 are presented the tissue/body sites of TCTEX1D4 interacting proteins expression pattern.

Table 6. Expression pattern of TCTEX1D4, PPP1CC and PRM2. Black color represents the highest transcription value and the lightest color, represents the lowest value of transcription.

TISSUES/BODY SITES	PROTEINS		
	TCTEX1D4	**PPP1CC**	**PRM2**
Bladder			
Blood			

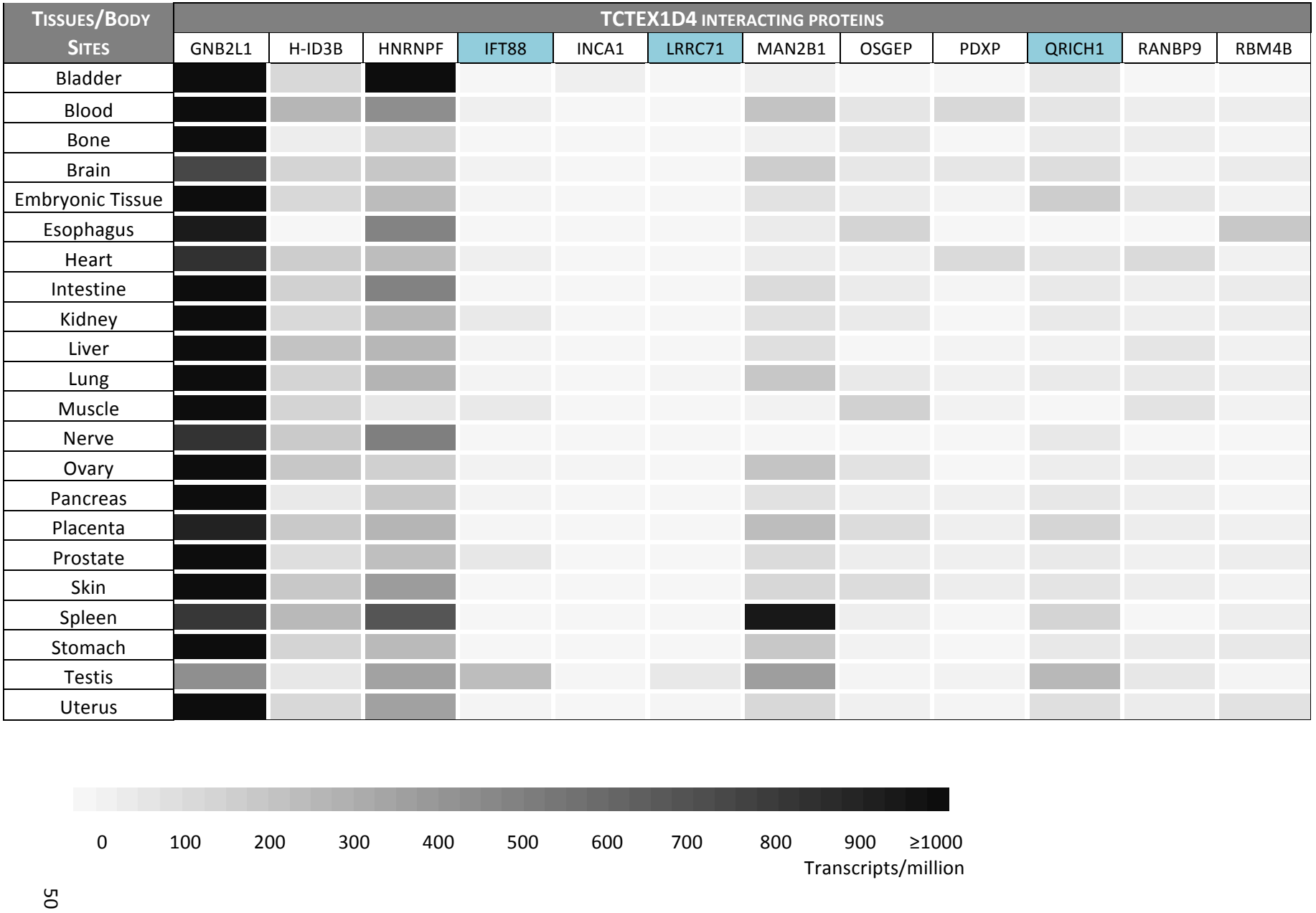
Tissues/Body Sites	Proteins		
	TCTEX1D4	PPP1CC	PRM2
Bone			
Brain			
Embryonic Tissue			
Esophagus			
Heart			
Intestine			
Kidney			
Liver			
Lung			
Muscle			
Nerve			
Ovary			
Pancreas			
Placenta			
Prostate			
Skin			
Spleen			
Stomach			
Testis			

0 100 200 300 400 500 600 700 800 900 ≥1000

Transcripts/million

Table 7. Expression pattern of TCTEX1D4 interacting proteins. The expression values are represented in a grayscale. Black color represents the highest transcription value and the lightest color, represents the lowest value of transcription. In green are highlighted the testis specific proteins, in blue the testis enriched proteins and in purple TCTEX1D2 a described flagellar protein.

TISSUES/BODY SITES	TCTEX1D4 INTERACTING PROTEINS											
	ACTN1	ATXN7L1	CCDC89	CRISP2	CTSB	CTSL1	FBLN1	FLOT1	FNDC8	GALNTL2	GARS	GEMIN4
Bladder												
Blood												
Bone												
Brain												
Embryonic Tissue												
Esophagus												
Heart												
Intestine												
Kidney												
Liver												
Lung												
Muscle												
Nerve												
Ovary												
Pancreas												
Placenta												
Prostate												
Skin												
Spleen												
Stomach												
Testis												
Uterus												


TISSUES/BODY SITES
TCTEX1D4 INTERACTING PROTEINS
GNB2L1
H-ID3B
HNRNPF
IFT88
INCA1
LRRC71
MAN2B1
OSGEP
PDXP
QRICH1
RANBP9
RBM4B
Bladder
Blood
Bone
Brain
Embryonic Tissue
Esophagus
Heart
Intestine
Kidney
Liver
Lung
Muscle
Nerve
Ovary
Pancreas
Placenta
Prostate
Skin
Spleen
Stomach
Testis
Uterus
0
100
200
300
400
500
600
700
800
900
≥1000
Transcripts/million

Tissues/Body Sites	TCTEX1D4 interacting proteins											
	SECISBP2	SMURF1	STAM2	TCTEX1D2	TERC	TIM50	TSC21	TTC28	UBE2D2	UQCRC1	USP33	WDR73
Bladder												
Blood												
Bone												
Brain												
Embryonic Tissue												
Esophagus												
Heart												
Intestine												
Kidney												
Liver												
Lung												
Muscle												
Nerve												
Ovary												
Pancreas												
Placenta												
Prostate												
Skin												
Spleen												
Stomach												
Testis												
Uterus												

TISSUES/BODY SITES	TCTEX1D4 INTERACTING PROTEINS			
	ZIM2	ZNF335	ZNF562	ZNF638
Bladder				
Blood				
Bone				
Brain				
Embryonic Tissue				
Esophagus				
Heart				
Intestine				
Kidney				
Liver				
Lung				
Muscle				
Nerve				
Ovary				
Pancreas				
Placenta				
Prostate				
Skin				
Spleen				
Stomach				
Testis				
Uterus				

0 100 200 300 400 500 600 700 800 900 ≥1000

Transcripts/million

In Table 6 is possible to observe that according to UniGene, TCTEX1D4 is only expressed in the lung and placenta. Curiously, the work performed by Korrodi-Gregório demonstrates that TCTEX1D4 is expressed in human testis and in rat, is highly expressed in ovary, spleen, lung, placenta and kidney (Korrodi-Gregório, 2012). In opposite what happens with TCTEX1D4, PPP1CC appears to ubiquitous. This is expected because the information provided by UniGene, does not differentiates between isoforms and therefore is not possible to assert which isoform is being expressed in each tissue. However is well established that PPP1CC1 is expressed ubiquitous and PPP1CC2 is testis enriched and sperm specific (Cohen, 2002; Fardilha *et al.*, 2010; Korrodi-Gregório, 2012). PRM2, as expected, is highly expressed in testis and low or not expressed at all in other tissues. This pattern of expression is typical of a testis specific protein.

In Table 7 describes the expression patterns of all TCTEX1D4 interacting proteins. In green are highlighted CRISP2 and TSC21. The two proteins are described in UniGene, UniProt and in several published literature (Busso *et al.*, 2005; Yu *et al.*, 2007; Cohen *et al.*, 2008) as being testis specific proteins and the pattern of expression presented is similar to PRM2, further confirming specificity of these two proteins. GEMIN4, IFT88, LRRC71, QRICH1 and SECISBP2 (in blue) were classified as testis-enriched proteins, which means that they are expressed in high levels in testis but not exclusively. In order to a protein being labeled as a testis enriched protein, it must have more than 100 transcripts per million in this tissue and no more than 3 tissues should have higher levels of expression than testis. However LRRC21 presents a exception to this rule, since it presents 61 transcripts per million but in other tissues is practically 0 and is not described as a testis specific protein, although in the literature we consider it a testis enriched protein. Finally, TCTEX1D2 (in purple), although neither testis specific or enriched, is a member of the dynein family, more specifically the DLC family, like TCTEX1D4. TCTEX1D2 was identified in *Chlamydomonas* flagellum, more specifically in the inner arm I1 (DiBella *et al.*, 2004) and in human is expressed in several tissues, including testis. The remaining proteins are expressed either ubiquitous or tissue-enriched.

IV.4. Functional characterization of the human testis TCTEX1D4 Interactome

To better understand functional attributes of TCTEX1D4 and it´s interacting proteins, cellular function was retrieved from HPRD and GeneOntology. Functional distribution of TCTEX1D4 interacting proteins is depicted in Figure 13. The proteins were distributed in 17 categories.

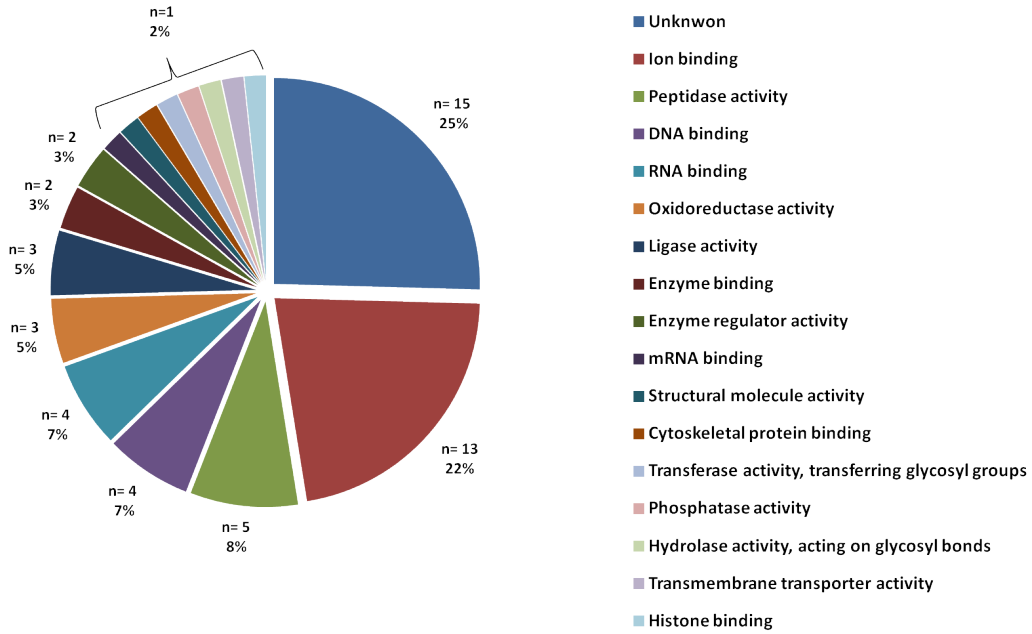


Figure 13 (previous page). Functional distribution of TCTEX1D4 interacting proteins. Functional categories were retrieved from GeneOntology. The same protein can be in different categories, due to have more than one function. Proteins that GeneOntology did not attribute a function were categorized as unknown.

The functional distribution of the identified proteins shows that TCTEX1D4 interacts with proteins involved in a variety of functions. This is not surprising, since TCTEX1D4 is a DLC and therefore may be involved in the retrograde transport of molecules. By controlling the subcellular localization of proteins, TCTEX1D4 can be indirectly responsible for their functions.

Also, in the graph two predominant groups are seen: proteins with ion binding function (22%) and proteins with unknown functions (25%). According to GeneOntology proteins with ion binding function interact selectively and non-covalently with ions, charged atoms or groups of atoms. Concerning the TCTEX1D4 interacting proteins with unknown functions they open novel avenues for further research, not only in the characterization of TCTEX1D4 function its self but also in the characterization of its interactors roles. Concerning testis, particularly male germ cells, all cellular function of TCTEX1D4 interacting proteins can be relevant to spermatogenesis. Besides ion biding proteins, which may be essential for the regulation of the concentration of ions, such calcium ions (Ca^{2+}) and therefore regulation of intercellular environment, proteins which binding DNA (7%) can be important for mitosis and meiosis, two processes crucial to spermatogenesis. Further, DNA binding, together with RNA binding (7%) and mRNA binding (1%) proteins are important for proper translation and transduction, a process that occurs in male germ cells only until spermatids. In latter spermatozoa, these proteins may no longer be necessary for spermatozoa physiology (Sutovsky *et al.*, 2006).

In spermatozoa , the fact that 25% of TCTEX1D4 interacting proteins appears to be involved in ion binding is very interesting, particularly, ACTN1 and FBLN1 that are specifically interacting selectively and non-covalently with Ca^{2+}, a ion involved in different phases of sperm function, such as sperm motility and acrosome reaction. In 2001 two groups reported the discovery of CatSper, a Ca^{2+} permeable cation channel that is only expressed in the principal piece of spermatozoa. CatSper deficient male mice present immotile sperm and therefore are infertile (Publicover *et al.*, 2011). In acrosome reaction studies with mouse proved that zona pellucida acrosome reaction is a Ca^{2+} dependent exocytotic event being an elevation of Ca^{2+} concentration required (Gupta *et al.*,

2011). The possibility of TCTEX1D4 being involved in Ca^{2+} regulation is promising and can unveiled important functions for this protein.

IV.5. TCTEX1D4 Network

To understand the interactions between TCTEX1D4 and the respective interacting proteins, the interactomes of all interactors were retrieved from PSICQUIC View (http://www.ebi.ac.uk/Tools/webservices/psicquic/view/main.xhtml). PSICQUIC View is a data retrieval tool, developed by the European Bioinformatic Institute (EBI, Cambridge, England) that standardizes the access to molecular interaction databases. The databases used to retrieve all the interactomes are listed in Table 8.

Table 8. Databases used to retrieve TCTEX1D4 interacting proteins interactomes. When performing a search in PSICQUIC View all databases listed are searched. Note that a search in PSICQUIC View does not mean that all databases produced results.

DATABASES			
APID	GeneMania	MatrixDB	Spike
BIND	I2D	MBInfo	STRING
BindingDB	InnateDB	Mint	TopFind
BioGrid	IntAct	MolCon	UniProt
ChEMBL	Interoporc	MPIDB	VirHostNet
DIP	IRefIndex	Reactome	

The interactions present in databases were detected by different methods. The predominant methods are biochemical techniques and YTH. Bioinformatic methods such as text mining and human based methods, like interactions inferred by curator are also present.

After all interactomes were retrieved, Cytoscape™ was used to fuse all interactomes, except for LRRC71, whose interactome was not available in any database. Besides, TCTEX1D4 interacting proteins, PPP1CC testis interactome was also fused. The interactome was obtained in the Signal Transduction Laboratory by preforming YTH screens with 3 different baits: PPP1CC1, PPP1CC2 and PPP1CC2end (the specific-terminal of PPP1CC2). TCTEX1D4 was identified as a PPP1CC binding protein in all screens and further confirmed by protein overlay (Fardilha *et al.*, 2011b) validating the YTH screen. Since this work focus on TCTEX1D4 interacting proteins in testis, protein sub-networks were constructed with testis specific and enriched proteins, as described previously, plus PPP1CC interactome. Also, TCTEX1D2 was further characterize, since it is a flagellar protein

present in *Chlamydomonas* axoneme (DiBella *et al.*, 2004). TCTEX1D4 interactome and the sub-networks of CRISP2, GEMIN4, IFT88, QRICH1, SECISBP2, TSC21 and TCTEX1D2 are represented in Figure 14.

The complexity of TCTEX1D4 interactome may reflect the diversity of processes in which TCTEX1D4 is putatively involved in testis. The sub-networks are simpler and allow the identification of possible TCTEX1D4 roles. The construction of interactomes allows a more knowledge-based choice of important complexes in a tissue, in this case, testis. Normally, the protein complex choice is made by the number of times that a protein is identified. Although this method may reflect the abundance of a protein complex in testis, it also may lead to an underlook of important protein complexes just because they are identified few times in the screens.

As is possible to observe in Figure 14, TCTEX1D4 and PPP1CC, besides interacting directly, are bridged by Ran binding protein 9 (RANBP9), possibly forming a trimeric complex. RANBP9 (or RANBPM) is a scaffolding protein. These type of proteins are important modulators of several physiological functions, such as accessories to multiprotein complexes and signaling modulators. Although identified as a Ran-binding protein, RANBP9 does not contain the consensus Ran-binding domain. RANBP9 has been shown to interact with a high diversity of proteins and in several systems, such as, the immune and nervous systems. For example, it has been shown to regulate the processing of amyloid precursor protein (Murrin *et al.*, 2007; Puverel *et al.*, 2011). In testis, RANBP9 appears to be essential for spermatogenesis, since null mice presented very small seminiferous tubules and were almost devoid of sperm cells (undergoing apoptosis). In this mice spermatocytes were present but post-meiotic cells (spermatids) were absent, suggesting that instead of progressing through meiosis, RANBP9 null mice spermatocytes underwent apoptosis (proven by TUNEL assay) (Puverel *et al.*, 2011). Since RANBP9 interacts with both PPP1CC and TCTEX1D4, these proteins can be involved in regulating/modulating RANBP9 function, by reversible phosphorylation and subcellular localization control. Seven sub-networks were constructed. The sub-network of TCTEX1D4/CRISP2/ACTB/PPP1CC is represented in Figure 14A. This protein complex will be analyzed in more detailed below. The sub-network of PPP1CC/TCTEX1D4/TSC21 is illustrated in Figure 14B. TSC21, is a Testis-specific conserved protein of 21 kDa, expressed only in testis and epididymis. Although its function is still unknown, it appears to be important in the transition from round spermatids to elongating spermatids (Yu *et al.*, 2007). The interactions between these three proteins can help understand TSC21 function. Figures 14C, D, E, F represent the sub-networks of testis-enriched proteins. GEMIN4 is a

component of the survival of motor neurons protein; SMN is required for pre-mRNA splicing by serving in the regeneration of spliceosomes. Although, member of these complex, the function of GEMIN4 is still unknown (Charroux *et al.*, 2000); IFT88, intraflagellar transport protein 88, is a member of the multi-subunit of the intraflagellar transport and acts as an adapter between the motor proteins required for movement and the ciliary cargo proteins (Delaval *et al.*, 2011; Taschner *et al.*, 2012). It is not surprising that IFT88 interacts with TCTEX1D4 since both proteins appear to be involved in IFT; QRICH1, glutamine-rich protein 1, presents a caspase activation recruitment domain which suggests the involved in apoptosis and inflammation (http://www.uniprot.org/uniprot/Q2TAL8); SECISBP2, SBP2-encoding gene Sec insertion sequence binding protein 2, is involved in the incorporation of selenocysteine, a special aa coded by a stop codon (UGA), into proteins. This is only possible on the presence of evolutionary conserved structures and proteins, such SECISBP2 that is necessary for translation of selenoprotein (Papp *et al.*, 2007). In Figure 14G is represented de sub-network of PPP1CC/TCTEX1D4/TCTEX1D2, which will be analyzed below.

Both CRISP2 and TCTEX1D2 were chosen to be further analyzed because although CRISP2 is a well described testis specific protein, its function in testis and in spermatozoa is still unclear. TCTEX1D2, although not a testis specific protein was described only once in the literature as flagellar dynein LC (DiBella *et al.*, 2004).

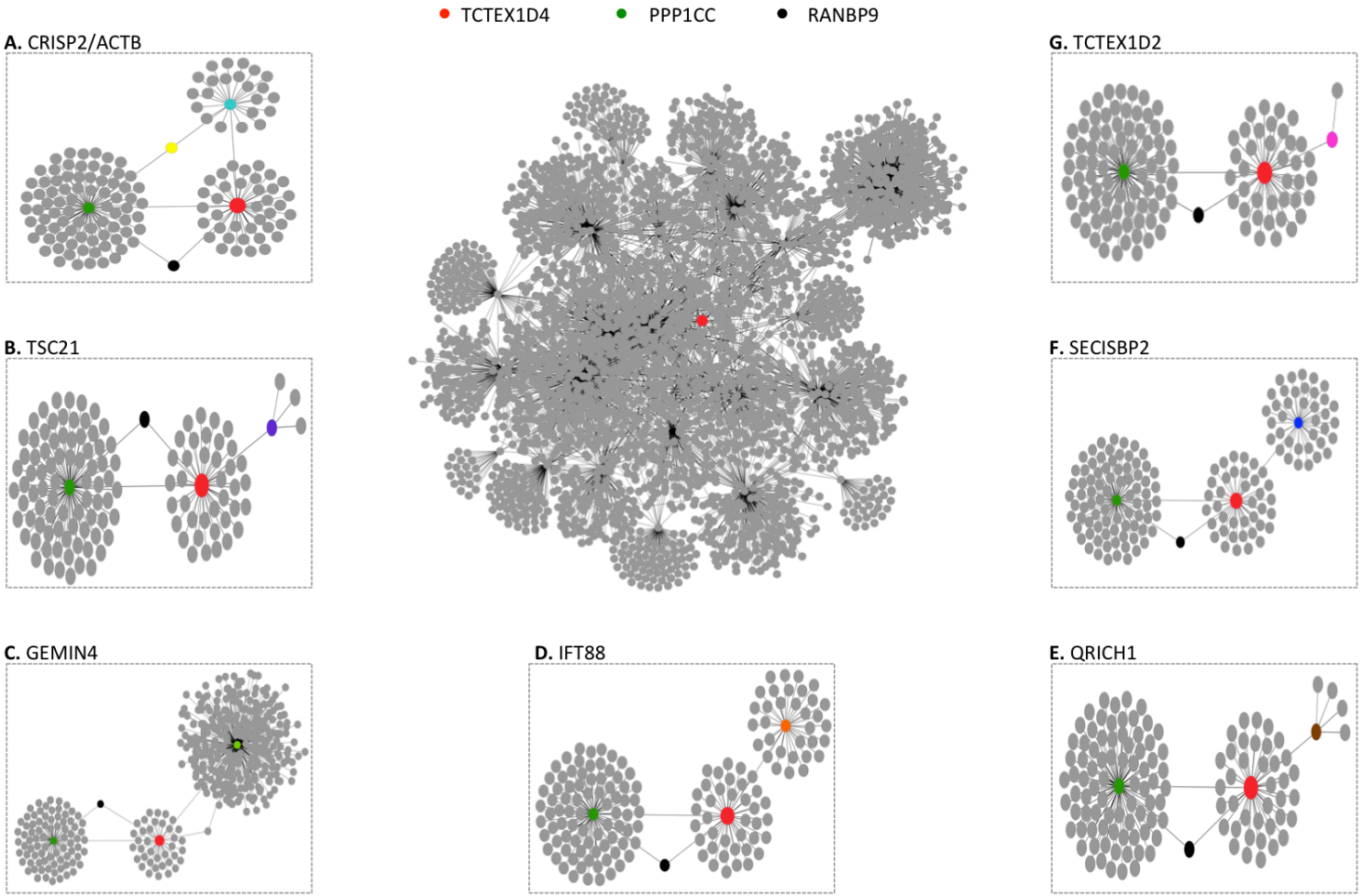


Figure 14. TCTEX1D4 interactome. In the center is TCTEX1D4 interactome. Surrounding this network is the testis specific and testis enriched protein interactomes. **A**. CRISP2 is in light blue and beta actin is in yellow. **B**. TSC21 is in purple. **C.** GEMIN4 is in light green. **D.** IFT88 is in orange. **E.** QRICH1 is in brown. **F.** SECISBP2 is in dark blue. **G**. TCTEX1D2 is in pink.

IV.6. TCTEX1D4/TCTEX1D2 complex

In the YTH system performed, partial sequencing identified TCTEX1D2 as a single positive clone, corresponding to the clone number 37. In Figure 15 is the nucleotide and corresponding amino acid sequence of TCTEX1D2 obtained using the ExPASy translate tool (http://web.expasy.org/translate/).

Figure 15. Nucleotide and amino acid sequence of TCTEX1D2. In green is marked the initiation codon; in red is marked the stop codon; in blue the PPP1BM present in TCTEX1D2 and in orange the nucleotide in which the insert started.

The clone retrieved was clone 37,2 that stars in nucleotide 28 (orange, Figure 15) and has 800kb in the agarose gel does, as only partial sequencing was performed we supposed the clone matches the data base sequence. Nevertheless, we cannot exclude the possibility of the C-terminus be different since in testis alternative splicing is a common mechanism (Fardilha *et al.*, 2011b).

TCTEX1D2 (T-complex testis expressed protein 1 containing domain 2) is a protein with 142 amino acids and a predicted molecular mass of 16,122 Daltons. It was described only once in the literature by DiBella et al (DiBella *et al.*, 2004). It was identified in *Chlamydomonas* flagellum and TCTEX1D2 defines a new subfamily of TCTEX2 DLC, the TCTEX1D2 subfamily, which also comprises an EST identified in human B cell lymphocytic leukemia, an EST present in human kidney and an

EST present in murine embryo. Is encoded by a single gene in the T-complex. As described earlier, flagellar dyneins comprise the inner and the outer arms. In *Chlamydomonas*, the inner arm system I1, who is responsible for the control of dynein motor function, is composed of 2 DHCs, 3 DICs and several DLCs, including LC8 and Tctex1. DiBella proved that Tctex1d2 is also a DLC in *Chlamydomonas* I1 inner arm. Tctex1d2 is up regulated in response to deflagelation, being drastically reduced only when *Chlamydomonas* strains are not able to assemble the inner arm I1. Also, it co-purifies with known components of inner arm I1, proving that this protein is an integral component of inner arm I1. However, it is not essential for inner arm I1 assembling, but to its stabilization. In the absence of Tctex1d2 (null mutant), inner arm I1 is unstable and in *situ* arm lacking Tctex1d2 displays deficiencies in motor function, being possible to observe nearly 25% reduction in microtubule sliding velocity relative to wild type. When reintroducing Tctex1d2 into the null strain, slides velocities were restored to the wild type. This indicates that Tctex1d2 is necessary for the flagellum function and therefore for motility (DiBella *et al.*, 2004).

Here we show that TCTEX1D2 interacts with TCTEX1D4 in human testis by the YTH assay. As TCTEX1D2 appears to be primarily localized to flagellar structures, it is possible that in human it is localized to the spermatozoon axoneme, specifically in the axonemal inner dynein arm, as it happens in *Chlamydomonas*. Furthermore, the TCTEX1D2 and TCTEX1D4 interactions further supports the flagellar localization of TCTEX1D4 as proposed by Korrodi-Gregório (Korrodi-Gregório, 2012). The fact that TCTEX1D4 and TCTEX1D2 form a protein complex may be an evidence that TCTEX1D4 is involved in sperm motility, as a component of the inner dynein arm. Curiously, TCTEX1D2 has orthologues in *Chlamydomonas* and in humans, but TCTEX1D4 is only present in vertebrates, maybe indicating a unique function for this protein (Korrodi-Gregório, 2012). One of the functions might be the binding to PPP1CC2 in spermatozoa, since both proteins appeared in the placental animals at the same time (Korrodi-Gregório, 2012). Interaction between TCTEX1D4 and PPP1CC may induce TCTEX1D4 dephosphorylation, since serine phosphorylation appears to be most relevant putative post-translational modification of TCTEX1D4 and consequently modifying TCTEX1D4/TCTEX1D2 interaction. Although PPP1CC2 is specific from spermatozoa, we cannot set aside that other phosphatases might be responsible for TCTEX1D4 dephosphorylation in spermatozoa (Korrodi-Gregório, 2012).

Taking into account TCTEX1D4 and PPP1CC localization in spermatozoa demonstrated by Korrodi-Gregório (Figure 16) and the TCTEX1D2 localization (DiBella *et al.*, 2004), there are two proposed models for TCTEX1D2/TCTEX1D4/PPP1CC2 complex. As higher PPP1CC activity is correlated to

sperm immotility in caput epididymis (Vijayaraghavan *et al.*, 1996; Fardilha *et al.*, 2011a), by contrary, it is possible that when TCTEX1D4 is phosphorylated, the TCTEX1D4/TCTEX1D2 complex is in a state where flagellar stability is promoted either by controlling/influencing/regulating TCTEX1D2 function and sperm is motile. In this case, an inhibitor such as PPP1R2 should repress PPP1CC activity (Fardilha *et al.*, 2011a). When PPP1CC is activated, TCTEX1D4 may be dephosphorylated and the interaction TCTEX1D4/TCTEX1D2 could change being TCTEX1D2 no longer capable of stabilize the flagellum, leading to immotile sperm cells. Note that as it happens in the *Chlamydomonas* flagellum, TCTEX1D2 in human is probably not essential for inner dynein arm incorporation in flagellum but increases the stability and enhances the performance of inner arm. Therefore, the flagellum integrity may not be compromise and thus, the sperm morphology will probably not change.

Also interestingly, TCTEX1D2 lacks any predicted phosphorylation site (DiBella *et al.*, 2004), so functional changes can be dependent on protein interactions, withTCTEX1D4, for instance, and TCTEX1D4 phosphorylation state may regulate TCTEX1D2, instead of direct post-translation modifications. However, other post translation modifications, like glycosylation can be present in TCTEX1D2 and be responsible for functional changes. Besides reversible phosphorylation of TCTEX1D4, PPP1CC can dephosphorylate intermediate chains and/or intermediate light chains, such as DYNC1LI1, DYNC1LI2 and IC2 (DiBella *et al.*, 2004). Curiously, although TCTEX1D2 cannot be phosphorylated, it has one PPP1BM, FQQRFR (35-40 aa) and therefore can be important for binding PPP1CC and presenting PPP1CC to other proteins.

Another putative function of the TCTEX1D2/TCTEX1D4 complex could be the cargo binding in intraflagellar transport, namely PPP1CC2. The complex could be an important retrograde PPP1CC2 transporter, moving this protein to specific motility-related PPP1CC2 targets. However, more plausible is that PPP1CC2 is important to the bind/release of cargos of the dynein motor unit, by controlling the phosphorylation state of TCTEX1D4 or/and the cargos themselves. This is supported by the fact that both DLC proteins have a PPP1BM (Korrodi-Gregório, 2012) being thus possible to assume that both can bind to PPP1CC2. In Figure 16 an illustrative diagram for the possible functions of TCTEX1D2/TCTEX1D4/PPP1CC complex is presented in intraflagellar transport and in regulation of sperm motility.

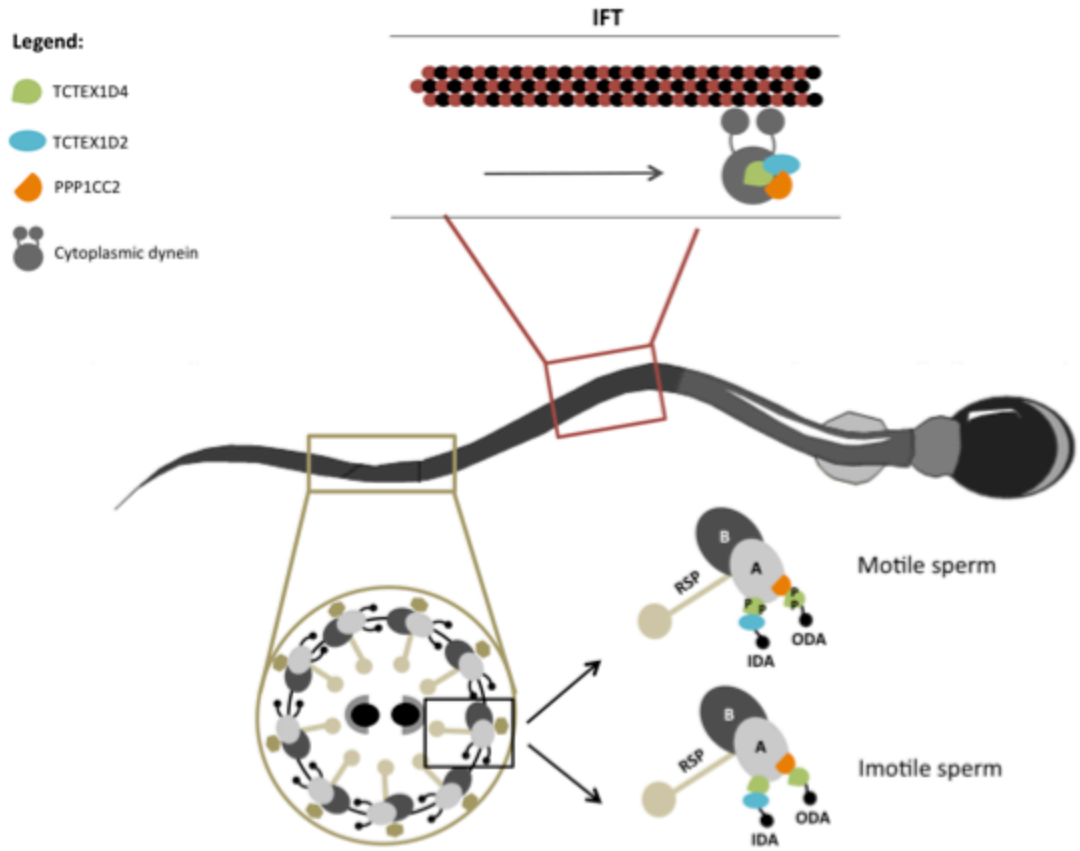


Figure 16. Putative functions of TCTEX1D2/TCTEX1D4/PPP1CC2 complexes in sperm. The protein complex can be involved in intraflagellar transport and in sperm motility. In intraflagellar transport, TCTEX1D2/TCTEX1D4 can be implicated in PPP1CC localization or/and PPP1CC can change the phosphorylation state of TCTEX1D4 or/and dynein cargos therefore important to dynein function. In axoneme, TCTEX1D4 can be involved in sperm motility by regulating axonemal stability through its interaction with TCTEX1D2 and alteration of its phosphorylation state by PPP1CC.

To better understand the TCTEX1D2/TCTEX1D4/PPP1CC complexes, future work will be necessary to provide answers to the functional importance of TCTEX1D4 in sperm physiology. However, TCTEX1D4 appears to have a putative role in motility acquisition in sperm cells through its function as an axonemal dynein.

IV.7. TCTEX1D4/CRISP2 complex

One protein identified as a TCTEX1D4 interacting protein in the YTH system, was CRISP2. Four independent clones were retrieved (clones 35,1, 35,2, 38 and 41), being 1 out of frame and 3 in frame. In Figure 17 is depicted CRISP2 nucleotide and corresponding amino acid sequence that was obtained using the ExPASy translate tool (http://web.expasy.org/translate/). The longer clone retrieved was clone 35,2 that stars in nucleotide 452 (orange, Figure 16) and has 800kb in the

agarose gel does, as only partial sequencing was performed we supposed the clone matches the data base sequence. Nevertheless, we cannot exclude the possibility of the C-terminus be different since in testis alternative splicing is a common mechanism (Fardilha *et al.*, 2011b).

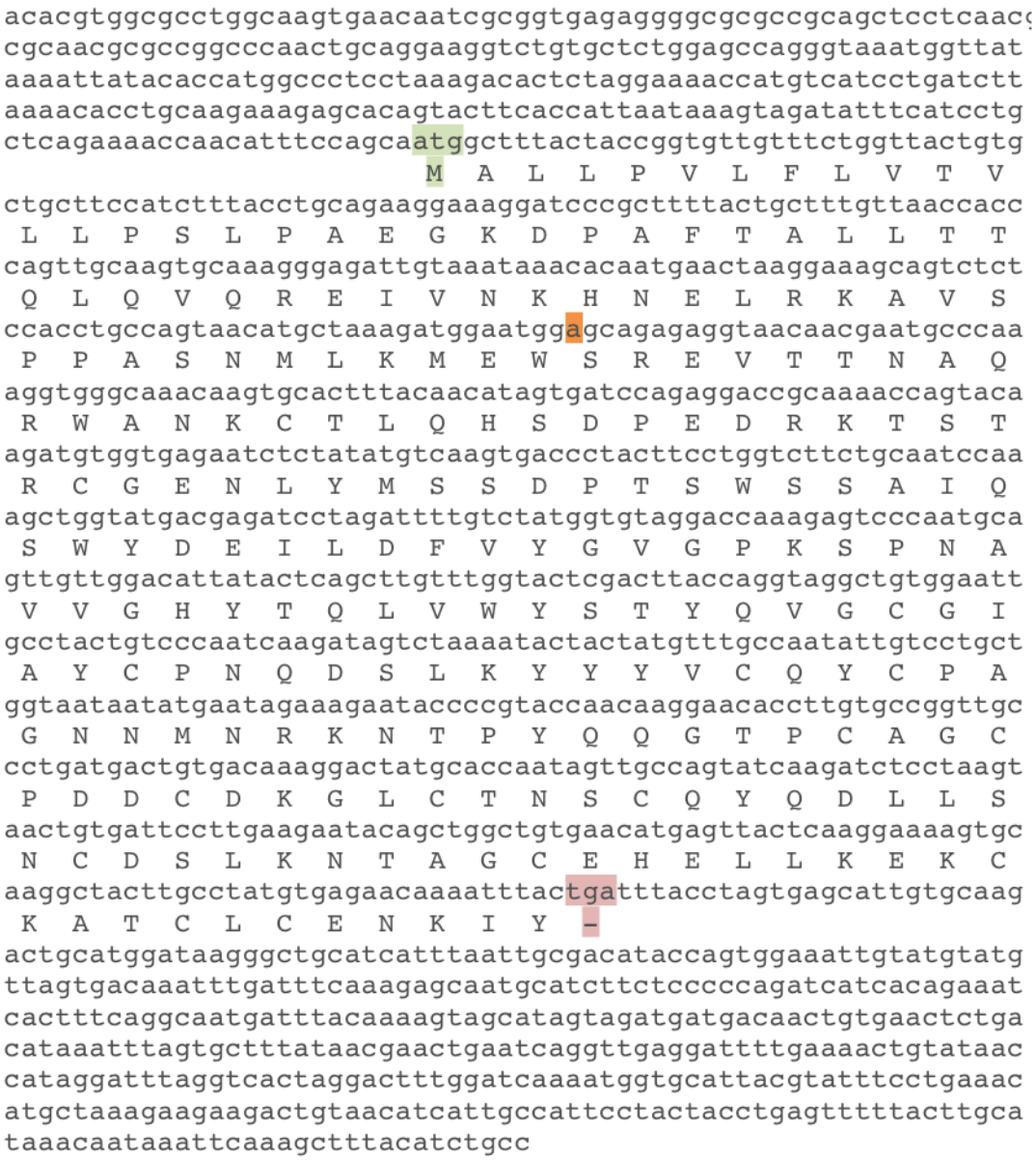


Figure 17. Nucleotide and amino acid sequence of CRISP2. The initiation codon is marked in green, the stop codon in red and in orange the nucleotide in which the insert started. The nucleotide sequence corresponds to the transcript variant 1 of CRISP2.

CRIPS2 (Cystein-rich secretory protein 2, also named TPX1) is a cysteine-rich secretory protein specifically expressed in the male reproductive tract, particularly in testis and in sperm cells. It

belongs to the wider CRISP family, composed of CRISP1, involved in sperm-egg fusion, CRISP2, CRISP3, present in reproductive and non-reproductive organs and CRISP4, expressed in the epididymis (Cohen *et al.*, 2011). Interestingly, significant molecular similarities have been unveiled between CRISP proteins and allergens present in salivary secretions of certain snakes and lizards and pathogenesis-related proteins from plants. (Busso *et al.*, 2005; Cohen *et al.*, 2007). All CRISP family members are characterized by the presence of 16 conserved cysteine residues. Of those, 10 are located in the C-terminus region (Da Ros *et al.*, 2008). Crystallographic analysis of several CRIPS family members revealed that they have 2 domains. The N-terminus region contains the plant pathogenesis-related domain (PR-1) and the C-terminus region contains the cysteine-rich domain (CRD) that are connected by a short hinge (Cohen *et al.*, 2007). Within domain PR-1 resides 2 small regions, signature 1 (S1) and signature 2 (S2) (Ellerman *et al.*, 2006; Cohen *et al.*, 2007). The PR-1 domain appears to have both protease activity and membrane-interacting activity and the S2 is the egg-binding site of CRISP2. CRD domain includes an ion channel regulatory activity (Ellerman *et al.*, 2006). A scheme illustrating the structural domains of the CRISP members family is shown in Figure 18.

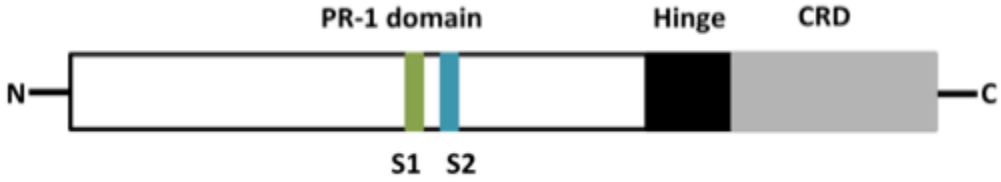


Figure 18. CRISP2 Structural domains. CRISP2 contains a plant pathogenesis related (PR-1) domain and a cysteine-rich domain (CRD), connected by a short hinge. Signature 2 (S2) and Signature 1 (S1) are located within the PR-1 domain. S2 is responsible for egg-binding capacity of CRISP2.

CRISP2 has 243 amino acids and a predicted molecular mass of 27,259 daltons. Although there are 6 different transcripts all encode the same protein. It was already shown that CRISP2 is involved in the fertilization process in several levels and perform different functions at different stages of sperm cell development (Busso *et al.*, 2005). This protein is present in the acrosome of sperm cells (Hardy *et al.*, 1988; Busso *et al.*, 2005; Busso *et al.*, 2007; Cohen *et al.*, 2007; Cohen *et al.*, 2008), in the out dense fiber in sperm axoneme (O'Bryan *et al.*, 1998) and in cell adhesion between spermatogenic and Sertoli cells in testis (Maeda *et al.*, 1998; Maeda *et al.*, 1999).

For fertilization to occur is necessary sperm-to-egg interactions mediated by proteins present in both gametes (Cohen *et al.*, 2008). In human and mouse sperm, CRISP2 is an intra-acrosomal

protein that remains in sperm acrosome even after the acrosome reaction, in the equatorial segment (Busso *et al.*, 2005). These behavior plus the fact that antibodies against CRISP2 diminish the percentage of penetrated oocytes, suggests a role of the protein in fertilization specifically gamete fusion through interaction with complementary sites on the egg surface (Busso *et al.*, 2007; Cohen *et al.*, 2008). Besides acrosome, CRISP2 is also present in the axoneme, specifically in the outer dense fiber (O'Bryan *et al.*, 1998). This flagellar component appears to be involved in the maintenance of the passive elastic structure of the flagellum and possibly in the regulation of flagellar motility, due to its high variable level of phosphorylation (see Figure 4) (Gagnon *et al.*, 2006). Finally, CRISP2 is also present in testis, where it is partially secreted (it presents a signal peptide, from aa 1 to 21: MALLPVLFLVTVLLPSLPAEG) from spermatogenic cells and acts as a bridging molecule so that spermatogenic and Sertoli cells functionally interact with each other (Maeda *et al.*, 1998; Maeda *et al.*, 1999).

Curiously, as shown in Figure 14, CRISP2 and PPP1CC share a common binding partner that is the beta actin protein (ACTB). Actins are ubiquitously expressed and are a component of the cytoskeleton, a highly organized dynamic structure that enables eukaryotic cells to regulate activities in response to extrinsic stimuli or intrinsic demand. Beta actin is a non muscle actin that is constantly shifting from a monomeric to polymerized form and vice versa in responsive to cellular processes, like shape changing (Xiao *et al.*, 2007). In testis, ACTB is present in all type of cells and in spermatozoon is localized to the posterior region of the sperm head, neck, midpiece and main piece of the tail. Even more interesting, a direct interaction between PPP1 and beta actin in testis was only reported once in literature by our previous work (Fardilha *et al.*, 2011b). Traditionally, it is stated that PPP1CC is delivered to the actin cytoskeleton through several PPP1 binding proteins such as neurabin I and phostensin (Auerbach *et al.*, 2002; Oliver *et al.*, 2002; Kao *et al.*, 2007). Therefore, the interaction between PPP1CC and ACTB can be indirectly mediated through PPP1 binding proteins but the possibility that both proteins interact directly cannot be excluded.

Due to the variety of subcellular localization of CRISP2 in spermatozoa, several functions for the TCTEX1D4/CRISP2/ACTB/PPP1CC complexes can be proposed. In acrosome, CRISP2 and TCTEX1D4 are both localized in the equatorial segment. However, PPP1CC2 is localized in acrosomal region and in post acrosomal region and ACTB is also in the post acrosomal region (Clarke *et al.*, 1982; Busso *et al.*, 2005; Korrodi-Gregório, 2012). This leads to the assumption that at least in the acrosome, CRISP2/TCTEX1D4 are the only proteins to form a complex. One of the hypotheses for

CRISP2 equatorial segment localization after acrosome reaction is the "release and association" method. This hypothesis states that CRISP2 is released from the acrosome during the acrosome reaction and then it binds to the surface of the equatorial segment essential for sperm-egg interaction (Maeda *et al.*, 1999; Busso *et al.*, 2005). What is not clear is how CRISP2 is transported to the equatorial segment and binds to it. We propose that TCTEX1D4 is responsible for the transport of CRISP2 to or from the equatorial segment explaining the similar localization of both proteins.

In axoneme, localization of TCTEX1D4, PPP1CC, CRISP2 (outer dense fiber) and ACTB (present in the flagella, although the specific location is unknown) leads to the assumption that these proteins can form a tetrameric complex (Clarke *et al.*, 1982; O'Bryan *et al.*, 1998; Korrodi-Gregório, 2012). As was said previously, the outer dense fibers appear to be responsible for maintaining a passive elasticity of the flagellum and to protect it from shearing forces (O'Bryan *et al.*, 1998). However, the high and variable level of phosphoserines in its proteins suggests some yet undetermined role in sperm motility regulation. It is proven that each one of the nine microtubule doublets is attached with an outer dense fiber, although how this association is established is unknown. We proposed that this association could be due to the direct TCTEX1D4/CRISP2 interaction. Thus, TCTEX1D4 that is present in both outer dynein arm and inner dynein arm interacts with CRISP2 in the outer dense fiber when is necessary and helps to maintain flagellar stability through flagellar movement. However, a tetrameric complex, TCTEX1D4/CRISP2/ACTB/PPP1CC can also be formed. This protein complex can be responsible not only for microtubule-outer dense fiber association, but also for sperm motility by modification of phosphorylation state of the outer dense fiber and/or dynein arm components being PPP1CC the key protein. Putative complexes localization, as well as, possible relevant functions in sperm cell is illustrated in Figure 19.

Furthermore, in testis, Korrodi-Gregório et al showed that TCTEX1D4 is present in the cytoplasm of most cells of the seminiferous tubules (germ and Sertoli cells), particularly, near the cell-cell junctions (Korrodi-Gregório, 2012), as was also shown for CRISP2 (Maeda *et al.*, 1998; Maeda *et al.*, 1999). PPP1CC2 is mainly in the nucleus (Chakrabarti *et al.*, 2007; Vieira *et al.*, 2011) and ACTB is distributed through the cytoskeleton (Xiao *et al.*, 2007; Lie *et al.*, 2010). According to the subcellular localizations of the proteins, TCTEX1D4/CRISP2 appears to be the only possible protein complex in testis. However, other complexes cannot be excluded, for example, in different developmental stages. Through spermatogenesis, differentiating spermatogenic cells need to be

in close contact with Sertoli cells to ensure a proper spermatogenesis and a cyclic function of Sertoli cells (Maeda *et al.*, 1999). Signaling pathways appear to be in the center of this communication between cells by direct cell-cell contact and/or paracrine mechanism. Meada et al. showed that CRISP2 is at least partially responsible for the adhesion between spermatogenic and Sertoli cells. This is further supported by the fact that CRISP2 is partially secreted and that it remains associated with the surface of spermatogenic cells acting as a bridging molecule and

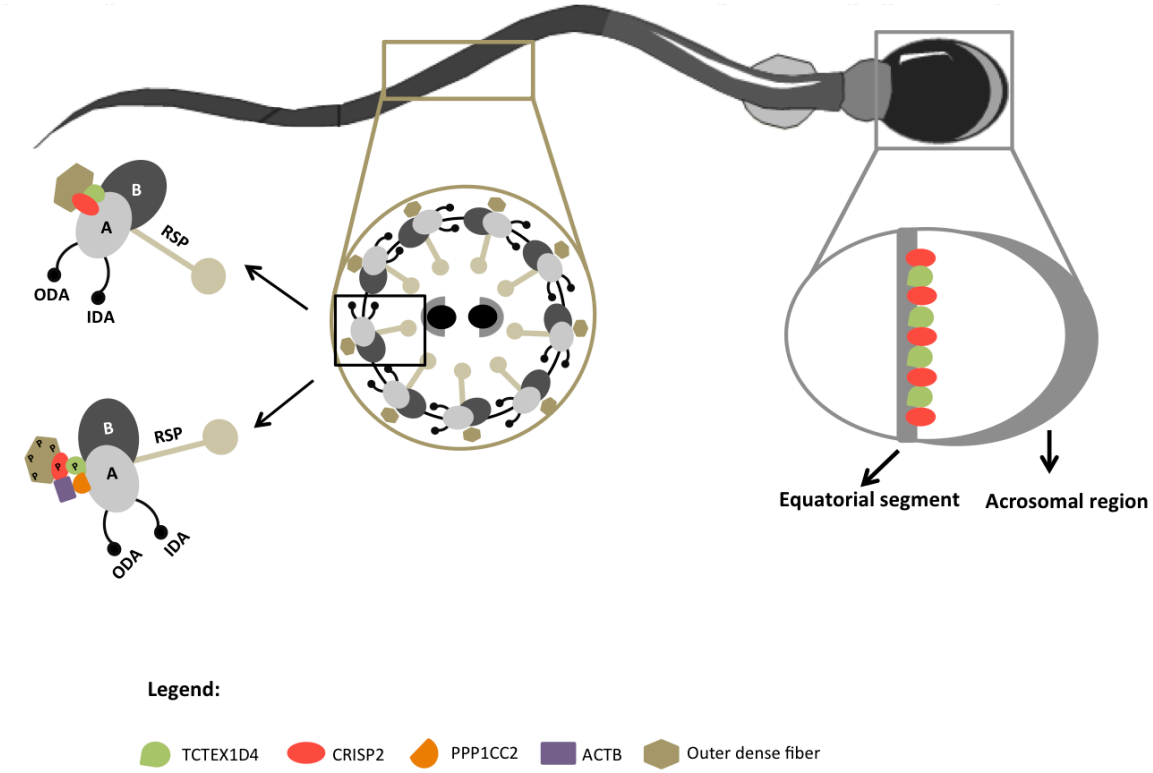


Figure 19. Putative functions of TCTEX1D4/CRISP2/ACTB/PPP1CC2 complexes in sperm. The protein complex can be involved in axonemal stability through connection of microtubule structure with outer dense fiber and in sperm motility through phosphorylation by PPP1CC2 of outer dense fiber and components including CRISP2 and TCTEX1D4 in the microtubule structure. In the acrosomal region, TCTEX1D4 can be involved in the transport of CRISP2 to or from the equatorial segment.

allowing functionally interaction between the cells. We propose that TCTEX1D4 is responsible for the retrograde transport of CRISP2 from the cell membrane to the center of the cell when association between the cells is disrupted and therefore being partially responsible for CRISP2 function by controlling it´s subcellular localization. In figure 20 is illustrated TCTEX1D4 and CRISP2 in testis and possible functions of protein complexes.

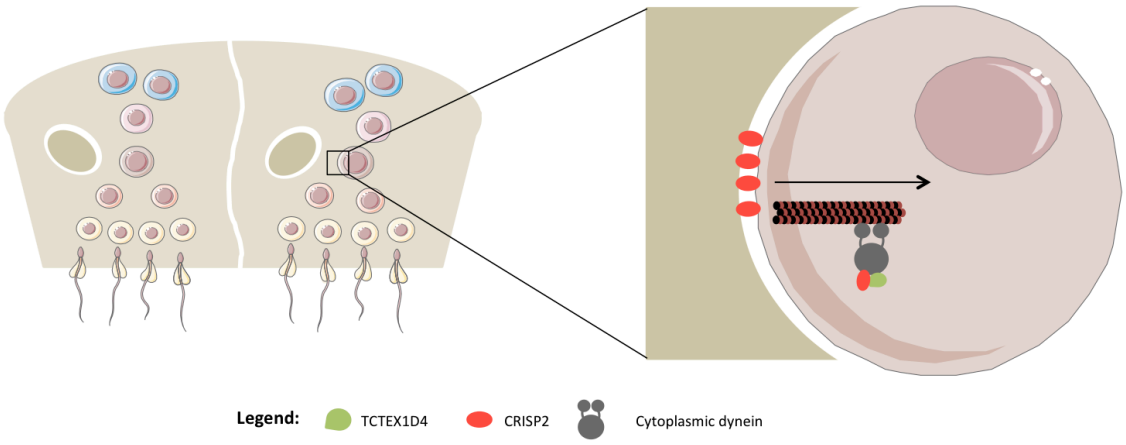


Figure 20. Putative functions of TCTEX1D4/CRISP2 complexes in testis. TCTEX1D4 appears to be involved in CRISP2 subcellular localization by retrograde transport of this protein.

All models proposed are only theoretical, although they are based in literature and the yeast two hybrid screen. Further work is necessary to confirm the relations and functions of TCTEX1D4 and its interacting proteins.

V. CONCLUSION

The YTH system is currently the most used method for detection of protein-protein interactions. It is based in the *Saccharomyces cerevisiae* genetic system and the transcription factor properties of eukaryotic cells. The YTH system has a number of advantages over other methods:it is an *in vivo* method and can be used to screen organism/tissue/cells (Bruckner *et al.*, 2009). In this work the YTH system was used to identify the TCTEX1D4 interactome, a PPP1 interacting protein. Eight six positive clones were identified, corresponding approximately ¼ of all positive clones obtained from the YTH human testis screen. To complete TCTEX1D4 interactome, the remaining positive clones will be identified in the future. Forty four different proteins were identified, being some of them identified more then once, for example INCA1 was retrieved 19 times. In the future a yeast colony hybridization must be performed to eliminate the most abundant cDNA inserts.

The results obtained suggest that TCTEX1D4 interacts with a variety of proteins involved in the most diverse cellular functions, such as, ion binding, phosphatase activity (as expected), peptidase activity and interestingly, unknown cellular functions (representing 15,25% percentage of protein functions). This is expected since TCTEX1D4 is a dynein light chain protein and therefore is prone to interact with a variety of molecules and move them through the cells. TCTEX1D4 may be responsible for other proteins function by controlling their localization within cell. However, further work is necessary to confirm TCTEX1D4 function and also the functions of some of it´s interacting proteins. An *in silico* tissue distribution profile of all TCTEX1D4 interacting proteins was performed with the purpose of identify testis specific and enriched proteins. Two specif testis proteins (CRISP2 and TSC21) and 5 testis enriched proteins (GEMIN4, IFt88, QRICH1 and SECISBP2) were identified. These specificity and enrichment must be confirmed by Western Blot and/or rt-PCR. To better understanding TCTEX1D4 interactome a protein network was constructed and the complexity of this network further confirmed the TCTEX1D4 promiscuity. Also, it was possible to identify putative key protein complexes in sperm physiology combining expression patterns and protein sub-networks of TCTEX1D4 interacting proteins.

CRISP2 a testis and sperm specific protein and TCTEX1D2 a flagellar protein were further characterized with the purpose of unveil possible specific TCTEX1D4 functions. When associated with TCTEX1D2, TCTEX1D4 may function as a retrograde transporter of PPP1CC in axoneme and/or as axonemal stabilizing protein complex. We also suggest that the phosphorylation state of TCTEX1D4, may be regulated by PPP1CC2, controlling the protein complex function.

When interacting with CRISP2, TCTEX1D4 can also function as retrograde transporter of CRISP2 in acrosome. In axoneme TCTEX1D4/CRISP2 interactions may be important to the interactions

between the dynein arm system and outer dense fiber and consequently in controlling the strength of the whipping move.

To confirm and validate TCTEX1D4 interactions with CRISP2 and TCTEX1D2, a co-imunoprecipitation and overlay studies can be performed for each protein. Also a colocalization study would be useful to assess *in situ* TCTEX1D4 interactions in acrosome and axoneme of sperm cell crossections.

Although much work is a head of us, the results here present unveil part of TCTEX1D4 interactome and also possible functions for TCTEX1D4 and were crucial to propose models for further studies. These results are the basis for many further studies based on each complex identified. This knowledge is essential to understand sperm physiology, primarily the acquisition of sperm motility. If we understand the processes responsible for sperm motility acquisition we can try to modulate it, inhibiting motility with the purpose of creating a male contraceptive or to treat male infertility

In conclusion, TCTEX1D4 interactome is functionally diverse and complex and it's characterization will reveal TCTEX1D4 functions in testis and spermatozoa.

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VII. APPENDIX

VII.1. Culture Media and Solutions

Yeast Media

10x Dropout solution

This solution contains all but one or more of the following components:

	10X concentration (mg/L)	**Sigma-Aldrich Química, S.A., Sintra, Portugal**
L-Isoleucine	300	I-7383
L-Valine	1500	V-0500
L-Adenine hemisulfate salt	200	A-9126
L-Arginine HCl	200	A-5131
L-Histidine HCl monohydrate	200	H-9511
L-Leucine	1000	L-1512
L-Lysine HCl	300	L-1262
L-Methionine	200	M-9625
L-Phenylalanine	500	P-5030
L-Threonine	2000	T-8625
L-Tryptophan	200	T-0254
L-Tyrosine	300	T-3754
L-Uracil	200	U-0750

10X dropout supplements may be autoclaved and stored for up to 1 year.

SD/QDO synthetic medium

To 800mL of deionised H_2O add:

5.36g Yeast nitrogen base without amino acids (Formedium Ltd., Norwich, England)

50mL glucose 40% solution (Sigma-Aldrich Química, S.A., Sintra)

Shake until the solutes have dissolved. Adjust the volume to 850mL with deionised H_2O and sterilize by autoclaving. Add glucose 50mL of a sterile 40% stock solution and 100mL of the appropriate 10X dropout solution.

Bacterial Media

LB (Luria-Bertani) medium with Ampicilin

To 950mL of deionised H_2O add:

LB 25g (MERCK, Darmstadt, Germany)

Agar 15g (for plates only) (MERCK, Darmstadt, Germany)

Shake until the solutes have dissolved. Adjust the volume of the solution to 1 liter with deionised H_2O. Sterilize by autoclaving. Add ampicillin (Sigma-Aldrich Química, S.A., Sintra) to a final concentration of 50μg/mL after LB cool down to 60ºC.

SOB medium

To 950mL of deionised H_2O add:

25,5g SOB Broth (Fluka, Sigma-Aldrich Química, S.A., Sintra)

Shake until the solutes have dissolved. Add 10mL of a 250mM KCl (Fluka, Sigma-Aldrich Química, S.A., Sintra) (prepared by dissolving 1.86g of KCl in 100 mL of deionised H_2O). Adjust the pH to 7.0 with 5N NaOH (MERCK, Darmstadt, Germany). Adjust the volume of the solution to 1000mL with deionised H_2O. Sterilize by autoclaving. Just prior to use add 5mL of a sterile solution of 2M $MgCl_2$ (Sigma-Aldrich Química, S.A., Sintra) (prepared by dissolving 19 g of $MgCl_2$ in 90mL of deionised H_2O; adjust the volume of the solution to 1000mL with deionised H_2O and sterilize by autoclaving) and 5mL of sterile solution of 2M $MgSO_4$ (prepared by dissolving 49.3g of $MgSO_4 \bullet 7H_2O$ in 90mL of deionised H_2O; adjust the volume of the solution to 1000mL with deionised H_2O and sterilize by autoclaving).

SOC medium

SOC is identical to SOB except that it contains 20mM glucose. After the SOB medium has been autoclaved, allow it to cool to 60ºC and add 20mL of a sterile 1M glucose (this solution is made by dissolving 18g of glucose in 90mL of deionised H_2O; after the sugar has dissolved, adjust the volume of the solution to 1 L with deionised H2O and sterilize by filtration through a 0.22-micron filter).

Competent Cell Solutions

Solution I

To 950mL of deionised H_2O add:

9.9g $MnCl_2 \bullet 4H_2O$ (Sigma-Aldrich Química, S.A., Sintra)

1.5g $CaCl_2 \bullet 2H_2O$ (Calbiochem, MERCK, Darmstadt, Germany)

150g glycerol (Calbiochem, MERCK, Darmstadt, Germany)

30mL potassium acetate 1M (Calbiochem, MERCK, Darmstadt, Germany)

Adjust the pH to 5.8, adjust the volume of the solution to 1000mL with deionised H_2O, sterilize by filtration through a 0.22-micron filter and store 4ºC.

Solution II

To 950mL of deionised H_2O add:

20mL 0.5M MOPS (pH 6.8) (Boehringer, Mannheim, Indianapolis, USA)

1.2g RbCl (Sigma-Aldrich Química, S.A., Sintra)

11g $CaCl_2 \bullet 2H_2O$

150g glycerol

Adjust the volume of the solution to 1000mL with deionised H_2O, sterilize by filtration through a 0.22-micron filter and store 4ºC.

Miniprep Solutions

Resuspension Solution

50 mM glucose

25 mM Tris-HCl (pH 8.0) (NZYTech Portugal, Lisboa, Portugal)

10 mM EDTA (Sigma-Aldrich Química, S.A., Sintra)

Lysis Solution

0.2 N NaOH

1% SDS (Amresco, Ohio, USA)

Neutralization Solution

3 M potassium acetate (Sigma-Aldrich Química, S.A., Sintra)

2 M glacial acetic acid (Sigma-Aldrich Química, S.A., Sintra)

Agarose Gel

Loading Buffer

0.25% bromophenol blue (Sigma-Aldrich Química, S.A., Sintra)

30% glycerol

50x TAE Buffer

To 950mL of deionised H_2O add:

242g Tris Base

7.1mL glacial acetic acid

100mL 0.5M EDTA (pH 8.0)Adjust the volume of the solution to 1000mL

Solutions

STET

8% Sucrose (NZYTech Portugal, Lisboa, Portugal)

5% Triton X-100 (Calbiochem, MERCK, Darmstadt, Germany)

50 mM Tris-HCl (pH 8,5)

50 mM EDTA

Ammonium Acetate 7.5M

To 80 mL of deionised H_2O add:

57.8g of Ammonium acetate (Sigma-Aldrich Química, S.A., Sintra)

Adjust the volume of the solution to 100mL with deionised H_2O and sterilize by filtration through a 0.22-micron filter.

Sodium Acetate 3M (pH 5.2)

To 950 mL of deionised H_2O add:

24.6g of sodium acetate (Sigma-Aldrich Química, S.A., Sintra)

Adjust the pH to 5.2 and adjust the volume of the solution to 1000Ml with deionised H_2O.

VII.2. Yeast Strains and Bacteria

E. Coli XL1-Blue: recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[F' proAB lacZΔM15 Tn10(Tet^r)]

Yeasts (Clontech, Saint Germain-en-Laye, France):

Strain	Genotype	Reporters	Transformation Markers
AH109	MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : GAL1UAS – $GAL1_{TATA}$-HIS3, GAL2UAS – $GAL2_{TATA}$-ADE2, URA3 : : MEL1UAS –$MEL1_{TATA}$-lacZ, MEL1.	HIS3, ADE2, MEL1, LacZ	Trp1, leu2

Y187	MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met–, gal80Δ, URA3 : : $GAL1_{UAS}$-$GAL1_{TATA}$-lacZ.	MEL1, LacZ	Trp1, leu2

VII.3. Plasmids

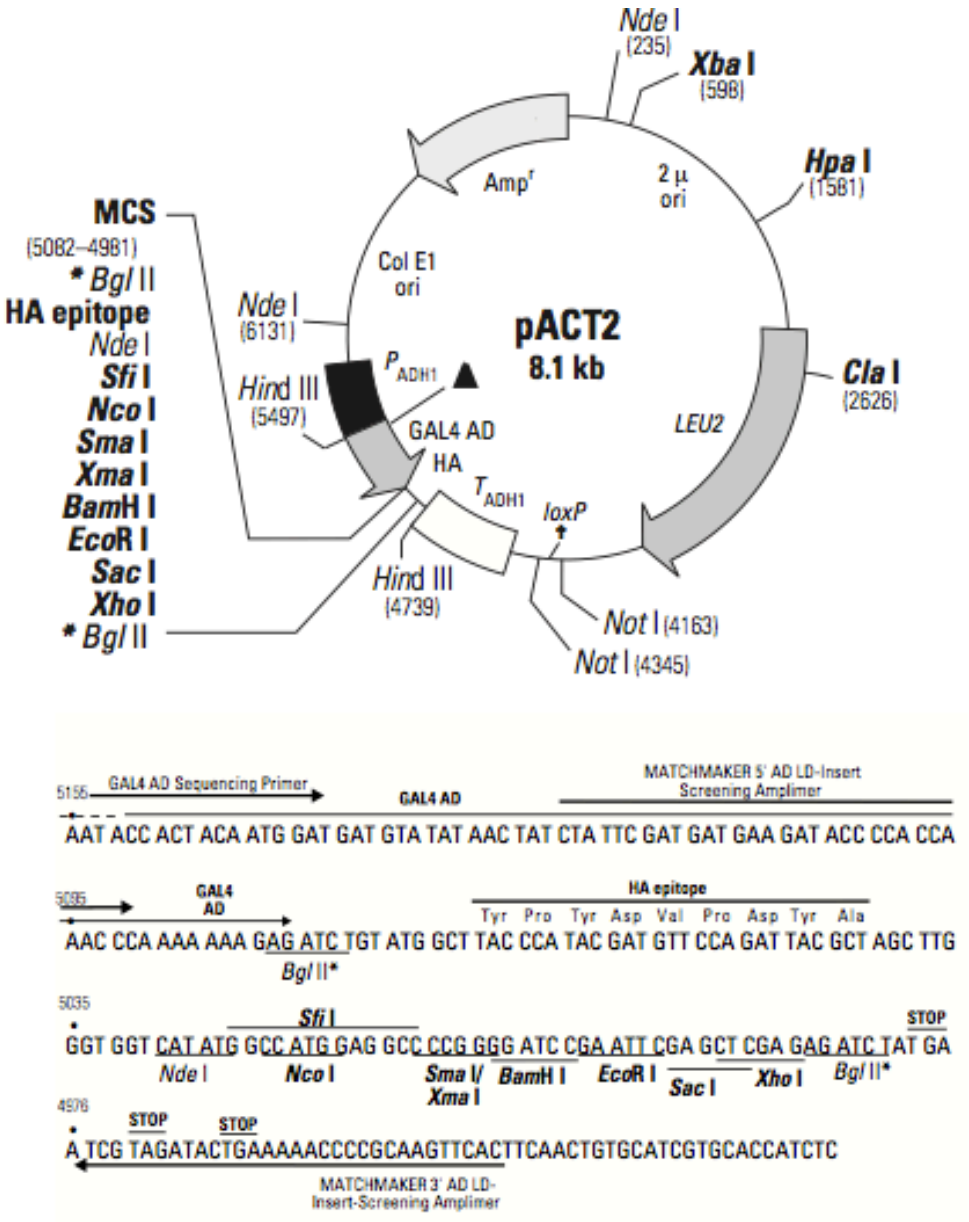

Figure 5. pACT2 map (Clontech, Saint Germain-en-Laye, France). pACT2 was used to generate a fusion of the GAL4 AD an epitope tag and protein enconded by a cDNA in a fusion library and is target to the nucleus by the nuclear localization sequence from SV40 T-antigen nuclear localization sequence. pACT2 I replicates autonomously in both *E. coli* and *S. cerevisiae* and carries the *bla* gene, which confers ampicillin resistance

in *E. coli.* pACT2 also contains the *LEU2* nutritional gene that allows yeast auxotrophs to grow in Leu *auxotrophic yeast strains.*

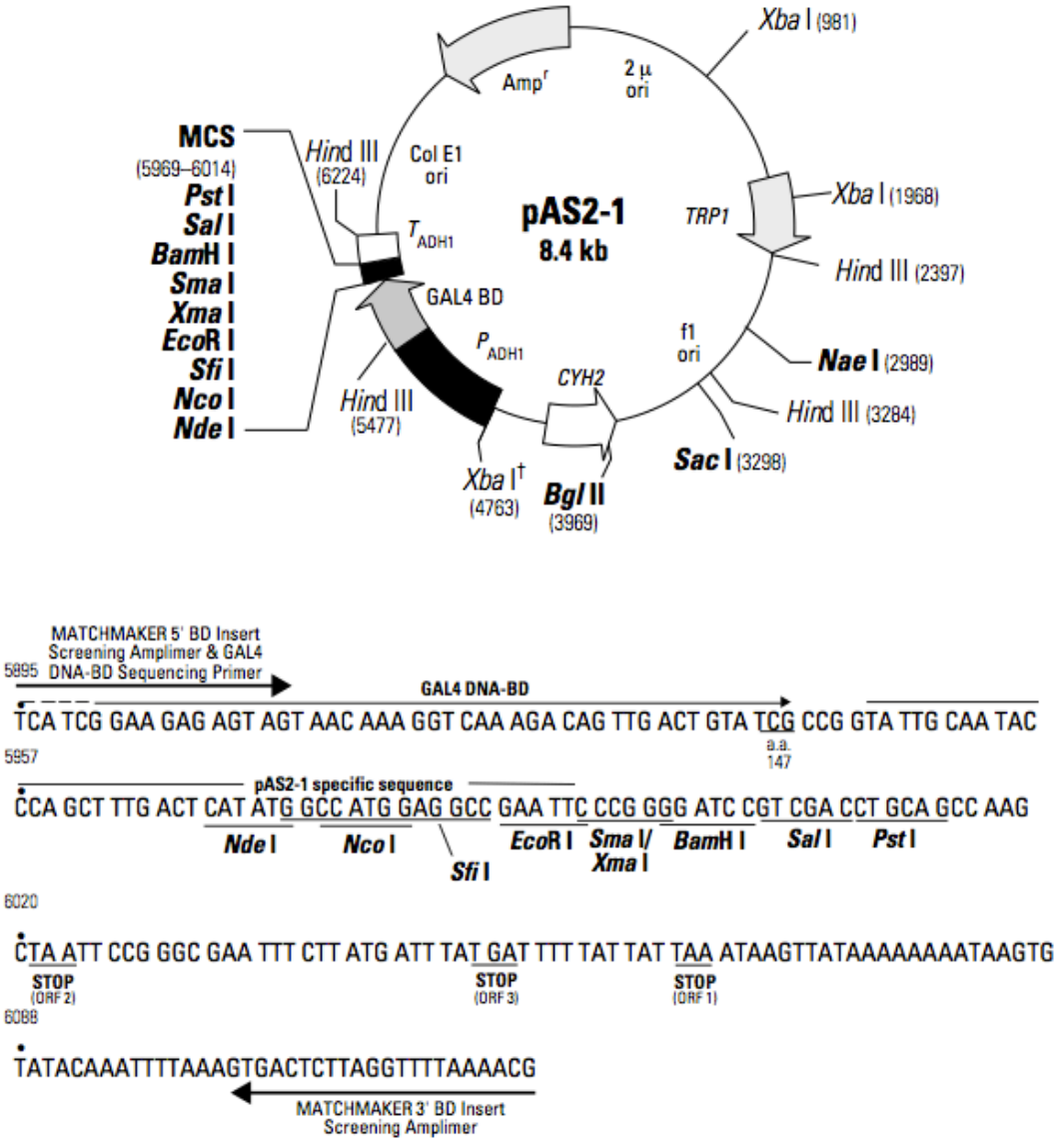

Figure 6. pAS2-1 map (Clontech, Saint Germain-en-Laye, France). pAS2-1 was used to generate a fusion protein of the GAL BD and TCTEX1D4. The hybrid protein is expressed at high levels in yeast host cells from the full-length ADH1 promoter. The hybrid protein is target to the yeast nucleus by nuclear localization sequences. pAS2-1 contains the TRP1 gene for selection in Trp- auxotrophic yeast strains.

VII.4. Primer

GAL4-AD Primer (Clontech, Saint Germain-en-Laye, France)

Sequence: 5´ TACCACTACAATGGATG 3´

Nucleotide number: 17

Melting Temperature: 48ºC